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ON THE CELL WALLS OF DIMORPHIC FUNGI CAUSING SYSTEMIC INFECTIONS[1]

BY F. BLANK[2]

Abstract

Cultures of the mycelial and tissue-like growth phases of *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Sporotrichum Schenckii* were extracted and oxidized as described by Scholl in 1908. Debye–Scherrer diagrams of the so prepared cell walls show the presence of chitin in both growth phases of each fungus investigated, and give no evidence of the presence of cellulose or another high polymeric substance in the membranes. Nitrogen determinations of the same material corroborate these findings. Electron-microscopic investigations of the isolated chitin of *Blastomyces dermatitidis* did not reveal any substantial difference in the submicroscopic structures of the framework of the mycelial and yeast-like growth phases.

Introduction

A number of species belonging to all classes of fungi may sometimes display dimorphic cultural characteristics; besides their normal mycelial growth form, a so-called yeast-like form of growth may appear. This phenomenon was first described by Bail (1), in 1857, then by Brefeld (5), and Pasteur (12) and attracted the attention of many mycologists of which only a few will be mentioned (3, 9, 18). Bainier (2), Molliard (11), and Frey (6) studied this phenomenon from the physiological and cytological point of view and found that the appearance of the yeast-like phase of growth is usually due to extreme ecological conditions.

Most of the fungi causing systemic infections in man and animals such as *Blastomyces dermatitidis* (North American blastomycosis), *Paracoccidioides brasiliensis* (South American blastomycosis), *Histoplasma capsulatum* (histoplasmosis), and *Sporotrichum Schenckii* (sporotrichosis) show a similar phenomenon. In the parasitic phase, the fungi causing these diseases are characterized by the absence of mycelium. The fungi occur in the host tissue as single yeast-like cells. All these fungi grow readily in culture in the mycelial phase at room temperature. Since yeast-like cultures closely resembling the parasitic phase have only been obtained in culture at 37° C., temperature has

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Contribution from the Department of Bacteriology and Immunology, McGill University, Montreal, Quebec. Presented as a paper at the Third Annual Meeting of the Canadian Society of Microbiologists, June 4-6, 1953, Guelph, Ontario.

[2] *Department of Bacteriology and Immunology, McGill University, Montreal, Quebec.*

been assumed to play an important role in the formation of this yeast-like form (10, 16). However, this biological phenomenon is far from being fully understood.

For this reason, the water insoluble polysaccharide of the cell walls of both growth phases of the pathogenic fungi mentioned before was isolated and identified in order to ascertain whether the framework of the cell walls consists of the same polysaccharide. An electron-microscopic investigation of the isolated polysaccharide was then undertaken to gain some insight into the submicroscopic structure of the substance forming the skeleton of the cell walls of both growth phases.

Materials and Methods

The mycelial phase of *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Sporotrichum Schenckii* was cultured in Erlenmeyer flasks on a medium consisting of 4% crude glucose, 2% Neopeptone "Difco", and 2% agar at room temperature. The yeast-like phase of *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* was grown on the same medium at 37° C., those of *Histoplasma capsulatum* and *Sporotrichum Schenckii* in the semisolid Y.P. medium as described by Salvin (14, 15) at 37° C.

The cultures grown on solid medium were killed by autoclaving at 121° C. for one hour, collected on a Buchner funnel from the warm liquid medium, washed several times with hot water to remove the adhering media, dried at 105–110° C., and ground in a mortar. Those grown in the Y.P. medium were also killed by autoclaving. The semisolid medium was then diluted by addition of water, the growth collected through a De Laval separator, and dried by lyophilization.

The dried material was then treated as described by Scholl (17), a method which we have previously employed in the investigation of the chemical composition of the cell walls of the dermatophytes (4). The material obtained in this way from the mycelial growth phase of these four organisms was yellowish-white, loose, and fluffy; that from the yeast-like forms was of the same color, but consisted of a coarse powder.

The yield of chitin isolated from these organisms varied from 2.5 to 5.1% of the dried weight whereas the chemically pure chitin obtained from the yeast-like phase of *Blastomyces dermatitidis* was 15.8%.

Microscopically, the treated material still showed all the morphological features as before extraction and oxidation. However, most of the cells of the yeast-like growth phase were broken up.

Debye–Scherrer diagrams of the extracted and oxidized material and the nitrogen determinations as well were performed in the same way as described before (4).

Results

(*a*) *Debye–Scherrer Diagrams*

The Debye–Scherrer diagrams obtained from the cell walls of both growth phases of *Blastomyces dermatitidis* were identical (Fig. 1).

PLATE I

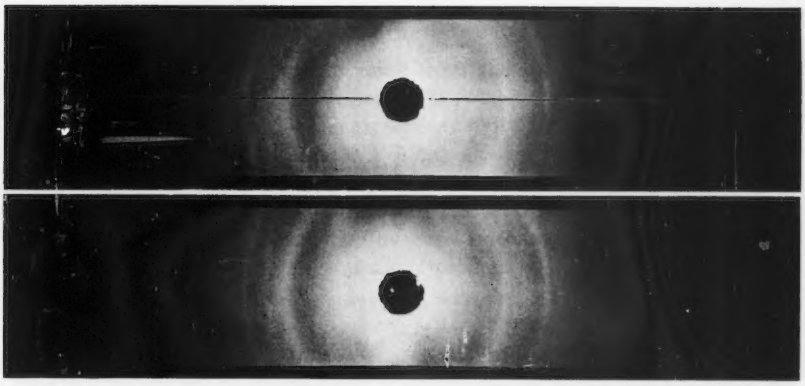

FIG. 1. Debye–Scherrer diagrams of the cell walls of *Blastomyces dermatitidis*. Above: mycelial growth phase. Below: yeast-like phase.

These two diagrams showed the typical powder pattern for chitin and were practically identical with other diagrams obtained from chitin isolated from other fungi (4, 7). The diffraction-lines of the diagrams were very large which indicated that the size of the crystals was less than 10^{-5} cm. The following spacings in Å-units were calculated approximately:

Blastomyces dermatitidis, mycelial phase

Intensity	Spacings in Å-units
Medium strong	9.8
Strong	4.7
Medium strong	3.4
Weak	2.4
Very weak	2.3

The Debye–Scherrer diagrams obtained from the specimens of both growth phases of *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Sporotrichum Schenckii* also revealed the presence of chitin; however, they contained a varying amount of two different inorganic impurities. One of these impurities was found to be corundum, the second one was believed to be tin oxide. Houwink and Kreger (8) reported the presence of quartz in cell wall preparations of yeasts boiled with dilute acid and subsequently with dilute alkali. They regard its presence as a contamination.

In none of the diagrams were there any lines for cellulose.

(*b*) *Nitrogen Content of the Cell Walls*

The results of the nitrogen determinations of the extracted and oxidized material as obtained by the Scholl method were as follows (Table I).

TABLE I

NITROGEN CONTENT OF THE CELL WALLS OF FUNGI CAUSING SYSTEMIC INFECTIONS

	Mycelial phase	Yeast-like phase
Blastomyces dermatitidis	6.14%	6.63%
Paracoccidioides brasiliensis	5.73%	6.38%
Histoplasma capsulatum	5.16%	5.84%
Sporotrichum Schenckii	5.50%	5.09%
Boletus edulis (17)	5.98%	
Agaricus campestris (13)	6.15%	
Pure chitin (20)	6.9%	

Owing to the scarcity of material further purification by the method of Zechmeister and Tóth (19) was impossible.

(*c*) *Electron Microscopic Investigations*

Electron micrographs of the chitin preparations of the mycelial and yeast-like growth phase of *Blastomyces dermatitidis* were made. Although different methods of preparation were used no substantial difference in the submicroscopic structure of the framework of the cell wall of these two growth forms could be ascertained.

Conclusions

The results of the Debye–Scherrer diagrams clearly indicate that the skeleton of the fungi investigated (mycelial and yeast-like phase) consists of chitin. These findings are corroborated by the nitrogen determinations.

It was shown in a previous paper (4) that the dermatophytes, in their parasitic phase, use the nitrogen-containing keratin for the synthesis of a nitrogen-containing polysaccharide, chitin, as skeletal material of their cell walls. Although the fungi causing systemic infections usually parasitize in tissue, a chemically different medium, the framework of their cell walls is also made of chitin.

The extremely different ecological conditions under which both growth phases develop do not influence the chemical composition of their skeletal material. They do not even induce a different submicroscopic texture of this skeletal material either in the mycelial or in the yeast-like phase as revealed by the electron-microscopic investigations. However, the different environmental factors have a morphogenetic effect, but this effect is formative only. The environment acts on the plasticity of the organisms causing different colonial appearances and different microscopic features of the mycelial and yeast-like growth phase.

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The author wishes to thank Prof. A. Frey-Wyssling, Zürich, for his kindness in having performed the electron-microscopic examinations in his laboratory, and Dr. W. Epprecht, Zürich, for the preparation and interpretation of the Debye–Scherrer diagrams.

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THE SUCCINOXIDASE SYSTEM IN *ASPERGILLUS NIGER*[1]

By S. M. Martin

Abstract

The succinoxidase system of *Aspergillus niger* differs from that of avian and mammalian tissues in its greater lability during preparation and storage but is similar in its sensitivity to malonate, cyanide, azide, and B.A.L., its association with cell particles, and its single pH optimum at pH 7.3. The system is not inhibited by arsenite in concentrations up to 0.01 *M*.

Introduction

If the terminal reaction in citrate synthesis is a condensation of "acetate" and oxalacetate, an adequate supply of the latter would be required for citrate accumulation in cultures of *Aspergillus niger*. The concept of citrate formation through a C_2–C_4 condensation is supported by studies of isotope distribution (2, 6, 8, 14) and fermentation products (18) and by the recent isolation of the "condensing enzyme" (12). Two separate mechanisms have been implicated in the formation of oxalacetate by *A. niger* (1, 2, 6, 8, 14):

I: pyruvate + carbon dioxide ⟶ oxalacetate

II: 2 acetate ⟶ succinate ⟶ fumarate ⟶ malate ⟶ oxalacetate.

This report deals with the succinate ⟶ fumarate reaction of the latter sequence.

Although conversion of succinate to fumarate has been demonstrated in *A. niger* (3, 17), there has been no detailed study of the enzymes concerned. The succinoxidase system of *A. niger* has, therefore, been investigated as a part of a general study of the mode of formation of citric acid by this organism. In undertaking this study it was decided to deal first with the enzymes of the "normal" mold, i.e. mold grown in a medium rich in all of the essentials for growth. Such mold accumulates little or no citrate, although the amount that is actually formed (and subsequently metabolized) may not differ greatly from that found in citrate yielding cultures. With such a basis for comparison it may then be possible to explain the accumulation of citrate by the abnormal or "pathological" type.

Methods

Aspergillus niger, N.R.C. 233, was grown in 200 ml. lots of a medium containing 3% malt extract, 0.5% yeast extract, and 1% glucose in 1-liter shake flasks at 26°. The growth (15–20 gm.) from two 18 to 20 hr. cultures was washed with water by filtration and the pressed pad of mold was then frozen in powdered dry ice. When completely frozen, the pad was ground for 30 min. in a mortar packed in dry ice, with twice its weight of acid-washed

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as N.R.C. No. 3292.

quartz sand and a little dry ice. Periodically during the grinding small quantities of dry ice were added to maintain the frozen state. On completion of the grinding, the mixture was allowed to thaw partially and a volume of phosphate buffer (0.1 *M*, pH 7.3–7.5) equal to twice the weight of the mold was added. After standing for 20 to 30 min., the mixture was centrifuged for one hour at 1000 r.p.m. This force was sufficient to remove unbroken cells and cell wall debris but not intracellular particulate matter. At no time was the temperature allowed to rise above 5°. The supernatant was removed and used immediately, except in experiments on the determination of pH optimum, when the preparation was dialyzed against four 4-liter lots of ice water for a total of one hour.

Enzyme activity was determined at 30° in the Warburg apparatus. Succinoxidase activity was measured in an atmosphere of air, and the oxygen uptake between 5 and 45 min. after the enzyme was tipped was used for the calculation of the $Q_{O_2}(N)$ (μl. O_2/hr./mgm.N). In the experiment illustrating the inhibitory action of British-anti-Lewisite (B.A.L.), succinic dehydrogenase activity was measured in an atmosphere of $N_2 : CO_2$ (95 : 5) using the ferricyanide technique (11).

The succinoxidase assay system contained: enzyme preparation, 0.5 ml.; phosphate buffer (0.3 *M*, pH 7.3), 1.0 ml.; potassium succinate (0.3 *M*), 1.0 ml.; cytochrome *c*, 0.64 mgm.; water, to 3.0 ml.; KOH (20%), 0.2 ml. in center well. The enzyme preparation was placed in the side arm of the vessel and was tipped after temperature equilibration which was carried out at the lowest shaking speed to avoid destruction of the enzyme (15).

The succinic dehydrogenase assay system contained: enzyme preparation, 0.5 ml.; bicarbonate buffer (0.16 *M*), 1.0 ml.; potassium succinate (0.3 *M*), 1.0 ml.; potassium ferricyanide (5 ml. 20% $K_3Fe(CN)_6$ + 1 ml. 0.16 *M* $NaHCO_3$), 0.2 ml.; water to 3.2 ml.

All the inhibitors except B.A.L. were added to the system at the beginning of the experiment; for B.A.L. the procedure recommended by Slater (15) was employed. The latter method involved incubation of inhibitor and enzymes preparation prior to dilution with other compounds of the assay system.

Fumaric acid was identified chromatographically using butanol – formic acid – water (5 : 1 : 4) as the solvent system (7).

Total Kjeldahl nitrogen was determined on each enzyme preparation using the microtechnique.

Results

The crude extracts prepared from *A. niger* rapidly took up oxygen when incubated in the presence of succinate but showed very little activity in the absence of added substrate. The data presented in Fig. 1 show the effect of varying the substrate concentration with other components of the assay system constant. Maximum oxygen uptake was obtained at a concentration of 0.02 *M* succinate under the conditions described.

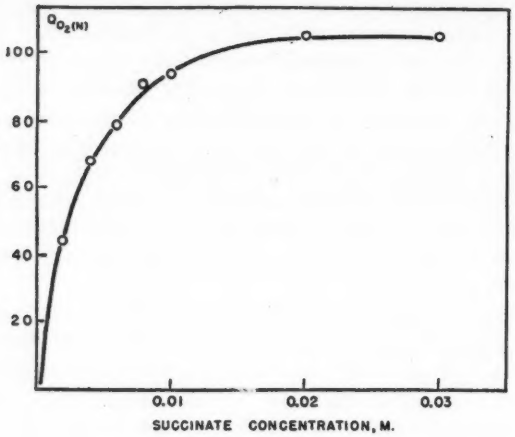

FIG. 1. Effect of substrate concentration.

When samples of the final reaction mixture, after incubation of the complete system, were chromatographed, the only acids detected were succinic and fumaric acids. Similar systems run in the presence of 0.01 *M* sodium arsenite gave identical chromatograms.

The crude enzyme preparations lost activity rapidly. In one experiment 93% of the succinoxidase and succinic dehydrogenase activity was lost on storage for 22 hr. at −1° and 57% was lost at −40° in the same period. This loss in activity can be assumed to have resulted from destruction of the dehydrogenase.

When the level of added cytochrome *c* was varied between 0 and 0.96 mgm. per vessel, the data shown in Fig. 2 were obtained. Although 66% of the

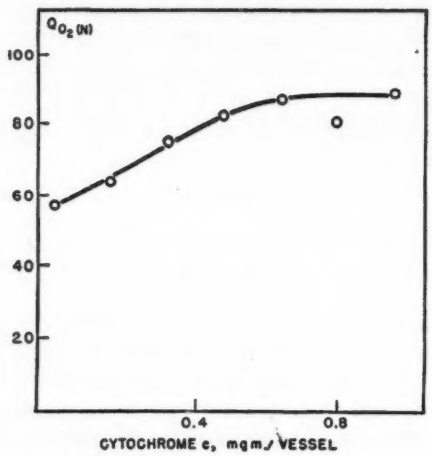

FIG. 2. Effect of added cytochrome *c*.

maximum activity was obtained in the absence of added cytochrome, attempts to demonstrate the presence of cytochrome using the Cary recording spectrophotometer failed. Not only were the 550 mμ and 521 mμ bands of reduced cytochrome *c* absent but the Soret or γ band was not detectable when crude extracts were examined spectrophotometrically. The latter band is characteristic of haematin compounds and of all of their derivatives with closed porphyrin rings (5).

The succinoxidase system of *A. niger* exhibited a single sharp pH optimum in the region of pH 7.3 (Fig. 3).

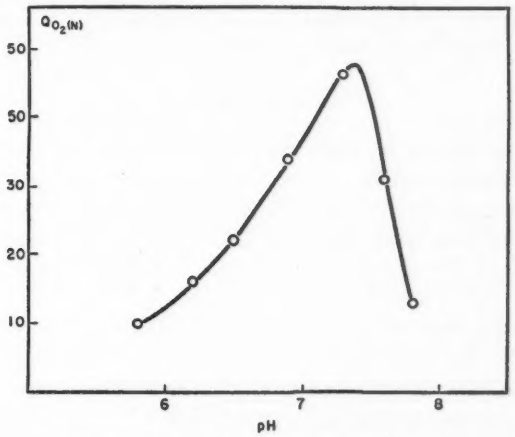

FIG. 3. Effect of pH (phosphate buffer).

The data in Table I show the effect of some inhibitors on the succinoxidase system of *A. niger*. Cyanide, azide, and malonate inhibited strongly whereas arsenite produced no inhibition in concentrations as high as 0.01 *M*.

TABLE I

EFFECT OF INHIBITORS ON SUCCINOXIDASE FROM *A. niger*

Inhibitor	Concentration, *M*	Inhibition, %
Cyanide	0.001	89
	0.010	100
Azide	0.001	36
	0.010	67
	0.050	100
Malonate	0.013	88
	0.030	100
Arsenite	0.001	0
	0.010	0

Slater (15) has shown that B.A.L. inhibits the succinoxidase system isolated from mammalian tissues but not the succinic dehydrogenase. A similar effect was observed by us with extracts from *A. niger*. In one series of experiments, for example, inhibition of the succinoxidase was 82% whereas the dehydrogenase was inhibited by only 11%.

The crude cell-free preparations of *A. niger* contained cell particles readily visible under low power magnification (225 ×). Centrifugation at high speed (35,000 r.p.m.) sedimented these particles and completely removed activity from the supernatant. Although the particles retained some succinoxidase activity when resuspended, the total activity recoverable was greatly reduced, presumably because of the lability of the system.

Discussion

The succinoxidase of *A. niger* differs from that of avian and mammalian tissues in its greater lability during preparation and storage but is similar in its sensitivity to malonate, cyanide, azide, and B.A.L., its association with cell particles, and its single pH optimum. In the latter respect it differs from succinoxidase of *Neurospora crassa* which has been shown (13) to have a double pH optimum (pH 6.9 and 8.1).

There is a difference of opinion concerning the effectiveness of arsenite as an inhibitor of the succinoxidase system. Potter and co-workers reported that succinate oxidation by chicken kidney homogenates was inhibited about 35% in the presence of 0.0033 *M* arsenite (10) and that the oxidation by rat liver homogenates was inhibited about 30% by 0.001 *M* arsenite (9). On the other hand, Green (4) has stated that 0.033 *M* arsenite permits oxidation of succinate in cyclophorase preparations. Szent-Györgyi (16) reported that 0.01 *M* arsenite did not appreciably inhibit succinoxidase activity in rat liver preparations. The succinoxidase of *A. niger* has been shown in the present paper to be insensitive to arsenite in concentrations up to 0.01 *M* and thus differs from the systems described by Potter and co-workers.

The inability to detect cytochrome in active preparations is difficult to explain since other data, such as stimulation by added cytochrome *c* and the effect of various inhibitors, clearly suggest the participation of the cytochromes in the oxidation of succinate. This is also at variance with the observation of Tamiya (17) who found a high cytochrome content in submerged hyphae of aspergilli. He also showed that surface mats are poor in cytochrome in spite of a high respiration rate. He explained these results by assuming that the respiration of surface cultures is mediated mainly by a flavin system whereas the cytochromes constitute the respiration mechanism in submerged hyphae. Shepherd (13) stated that the cytochromes have not been demonstrated in *Neurospora* in spite of an active succinoxidase system. It thus appears that further work will be required to elucidate the role of the cytochromes in the conversion of succinate to fumarate by molds.

Acknowledgment

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LISTERIA MONOCYTOGENES—A STUDY OF TWO STRAINS ISOLATED FROM HUMAN LISTERIOSIS[1]

By Robert F. Jaeger[2, 3] and Donald M. Myers[2]

Abstract

Studies on two strains of *L. monocytogenes* isolated from human patients, have been reported. The 1949 and the 1951 strains are serologically similar but have slight biochemical variations. Cultures grown at temperatures ranging from 2.8° C. to 5° C. showed greater virulence and antigenicity as well as morphological differences when compared with cultures grown at 37° C. Both the 1949 and 1951 strains were sensitive to penicillin in concentrations between 0.09 to 0.18 and 1.50 to 3.12 units per ml. respectively. They were not sensitive to sulphadiazine or sulphathiazole in concentrations up to 20 mgm. per ml. Both strains produced an antigenic (lipoid-like) surface substance when extracted with chloroform and ether, which, when injected into rabbits, produced a monocytic response in the peripheral blood. A heat-killed vaccine protected guinea pigs from lethal doses of the *Listeria* organism, whereas control guinea pigs injected with the same number of living organisms died with typical symptoms. Partial immunity is demonstrable by the fact that rabbits recovering following penicillin therapy resisted reinfection for a period of at least one hundred and ninety days. Further work is being carried on to determine whether there is a relationship between the heat-labile "L" surface antigen and virulence.

Introduction

Listeria monocytogenes was isolated from rabbits originally by Murray, Webb, and Swann (9) and given the name of *Bacterium monocytogenes* because of the increase in mononuclear cells which occurred in the diseased animals. Pirie (10) isolated the organism in a plaguelike disease from rodents of South Africa and named his strain *Listerella hepatolytica*. Subsequently, Gill (4) described a disease in sheep which was caused by this organism and even later it was described by other workers who showed it to be the etiologic agent of disease in many species of birds and mammals.

Little was known of the infection in man, primarily because of its rarity. Probably a number of early cases were wrongly classified as being caused by a "diphtheroid" since other corynebacteriaceae are morphologically similar to this organism. Schultz (11) made the first report of the isolation of a Gram positive rod from a patient suffering from meningoencephalitis and proved this rod to be *Listeria monocytogenes*. Burn (2) described four fatal cases of listeriosis in infants in which three had positive blood cultures and all showed focal necrotic areas of the liver. Later infections in man and animal have been reported from many areas, demonstrating that *L. monocytogenes* is of world-wide distribution. Occasionally it occurs in epidemic form in animals, in contrast to sporadic cases in humans having a syndrome resembling infectious mononucleosis. This paper deals with strains that were isolated

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The opinions expressed are those of the authors and do not constitute an endorsement by the Medical Department of the U.S. Navy.

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from two nonfatal meningitis cases at the U.S. Naval Hospital, Bethesda, Maryland, in 1949 and 1951. A case history involving the 1949 strain was reported by Berry (1). The purpose of this presentation is to give details of a comparative study of these two strains, with regard to their morphological, cultural, serological, and pathogenic characteristics.

Methods

Cultural and Biochemical Studies

In studies presented in this paper, human blood agar plates and trypticase soybean broth (BBL) with 1% dextrose added were used. Fermentation reactions were determined by using phenol red broth base (BBL) with 1% concentration of the carbohydrate and adjusting the final pH to 7.4. The fermentation tubes were inoculated from original cultures (isolated on blood agar) and incubated for 30 days at 37° C.

Serological Tests

Dilutions of 200,000 living organisms per ml. (determined by optical density) were injected intravenously into rabbits to obtain specific immune sera. At what appeared to be the critical stage of the disease penicillin* therapy was instituted and continued until the animal recovered. Test sera were taken at various intervals following recovery.

To demonstrate acquired immunity guinea pigs weighing 250 gm. were vaccinated. The vaccine was prepared by using organisms washed from blood agar plates with normal saline. This suspension was diluted to equal one billion organisms per ml. (optical density method), heat-killed in a water bath at 60° C. for one hour, and then checked for sterility. This vaccine was administered intraperitoneally in five increasing doses at intervals of four days ranging from 0.25, 0.5, 1.0, 1.5, and 2.0 ml. Two weeks after the final inoculation an injection of living organisms sufficient to kill unprotected animals was administered.

The antigens for testing were prepared from 24-hr. broth cultures, following the technic described by Hepler (6) for flagellar and somatic antigens. The presence of antibody response to these antigens was confirmed by agglutination involving 18 hr. incubation at 37° C. with final readings being made following overnight refrigeration.

Escherichia coli K8(08) strain No. G3404/41, known to contain the "L" antigen, was used to produce "L" antiserum for the study of heat-labile "L" antigen of *L. monocytogenes*, found to be present as a surface antigen by the author (DMM). Further research on this antigen is in progress and will be published at a later date. Sera and antigens were prepared using the methods of Kauffmann (8) and the confirmatory agglutination tests were incubated for two hours at 37° C. and refrigerated overnight before final results were recorded.

* *Schenley Aqueous Crystallin G.*

Pathogenicity Studies

Seven-month-old albino rabbits, guinea pigs, and white mice were used to determine pathogenicity since all three are selectively affected by *L. monocytogenes*. The living organism was injected intravenously into the rabbit but intraperitoneally and intracerebrally into the guinea pigs and the mice. The rabbit was used principally, because it develops clinical symptoms, with characteristic changes in blood cells.

Morphology, Cultivation, and Biologic Properties

Growth of *L. monocytogenes* is evident on ordinary media, such as nutrient agar, within 18 hr., but is more luxuriant on a medium to which 0.5% dextrose or 5% sheep or human blood has been added. Colonies on blood agar are small, translucent, milk white with a clear to hazy zone of hemolysis similar to that of beta streptococcus. The colonies may reach the size of 0.5 mm. in diameter. In broth there is a diffuse turbidity with sedimentation after 24 hr. incubation.

Rods: 0.5 by 1 to 2μ, Gram positive, rounded ends, occurring singular, in pairs, "V" shapes, and with tendencies toward chaining; nonspore-forming, nonencapsulated (?), aerobic to facultatively anaerobic.

Growth at 37° C.: sluggishly motile or nonmotile.

Semisolid medium stab: branched out growth at 18 to 24 hr. becoming diffuse after 48 hr. at 37° C.

Carbohydrates: both strains produced acid without gas in dextrose, salicin, rhamnose, levulose, maltose, glycerol, dextrin, and tartrate. (The 1949 strain did not ferment lactose, xylose, or galactose but did ferment arabinose and inositol.) No fermentation in sucrose, mannitol, dulcitol, inulin, sorbitol, or melezitose.

Gelatin: not liquified.

Hydrogen disulphide: negative.

Indol: not produced.

Urea: negative.

MR-VP: negative.

Nitrites: not produced from nitrates.

Litmus milk: acidified without coagulation.

The two *Listeria* strains were found to remain viable for long periods of time in either broth or on agar cultures. Broth cultures grown at both room and incubator temperatures for a period of seven months produced good growth within 18 hr. when subcultures were made. Smears made from the seven-month-old cultures showed little loss in their Gram positive reaction.

Gray *et al.* (5) had observed that isolation of the organism from an infected bovine brain was more successul if the brain had been stored in the refrigerator for one month. They felt that this was due to a brain cell breakdown releasing the organisms. The present research shows that moderately good growth can be obtained in 7 to 10 days from these two strains if inoculated broth cultures

are incubated at temperatures that range from 2.8° C. to 5° C. It is now felt that the factor responsible for the more successful isolation of the organism was the reduced temperature range rather than a cellular deterioration that had released the organisms. It should be noted that at this lowered incubation temperature certain morphological changes take place, i.e., (1) the length of the organism tends to increase from 1.5 to 4μ; (2) a greater amount of palisading and parallel formation becomes apparent; (3) there is noticeable granulation; and (4) short chain formation is evident. Capsule stains done on cultures incubated at 2.8° C. to 5° C. consistently showed a capsular-like material surrounding the cell, but this material was not apparent on cultures grown at 37° C. This material will be discussed in greater detail later.

Pathogenicity in Animals

Rabbits, guinea pigs, chick embryos, and white mice were employed to demonstrate pathogenicity in animals.

Pathogenicity for Rabbits

Large numbers of organisms injected intravenously caused a rapid death from septicemia with positive blood cultures, liver abscesses, and occasional congestion in the vascular spaces of the heart being demonstrated. Those animals receiving small numbers of the organism, (under 100,000/ml.) usually developed clinical symptoms but later showed an antibody response and survived.

A seven-month-old rabbit, No. 100, that had been isolated for one week prior to use, was injected with 1 ml. of a suspension containing 200,000 living organisms. Total white blood counts and differential leucocyte counts were done twice daily. For the first four days following injection, the rabbit exhibited no change in blood counts and no other signs of illness, except a rise in rectal temperature to 109.4° F. on the second day, which receded to 106° F. on the following day. On the fifth day there was a definite increase in mononuclear cells with some atypical lymphocytes having vaculation. On the 11th day it was necessary to stop blood counts because of the very marked leucopenia. At this time there were great numbers of premature erythrocytes in the peripheral blood stream as well as a decrease in hemoglobin content. The rabbit was found to be weak, listless, anorexic with labored respirations and severe diarrhea which resulted in a loss of 450 gm. in body weight from the time of inoculation. During the night of the 11th day following injection the rabbit died, and was autopsied. Smears and cultures were made of the organs; bacteria were demonstrated in the heart blood, heart tissue, liver, spleen, and kidneys.

Pathology

The liver showed a diffuse distribution of small, yellow focal zones of necrosis throughout. Microscopically, the liver revealed severe fatty metamorphosis, particularly about the central veins. Here the liver cells were vacuolated.

There was a considerable number of monocytes and atypical lymphocytes in the sinusoids, with an occasional megakaryocyte present. The heart was selectively affected with microabscesses composed of eosinophiles and monocytes. In these areas the myocardial fibers were necrotic and showed hyalinization with loss of cross and longitudinal striations. The heart differed from most of the other tissues by having a high phospholipid content which may have been the cause or result of localization. There was congestion in the red pulp of the spleen with a marked infiltration of plasma cells. Numerous hemosiderin-filled macrophages were noted indicating probable blood destruction. The kidneys were unremarkable except for slight passive congestion and clusters of eosinophiles and monocytes about the veins. There was, however, no brain involvement. The course of disease in this rabbit was similar to experimental rabbit infections reported by Burn (2).

Another seven-month-old rabbit, No. 200, was injected in the same manner as rabbit No. 100; this rabbit exhibited clinical symptoms similar to those found in rabbit No. 100 on the fifth day following injection. White blood counts and differentials were performed throughout the illness (Table I). At this time the rabbit appeared moribund; treatment consisting of twice daily intramuscular injections of 50,000 units of penicillin was started. There followed a relapse on the third day of treatment which was characterized by convulsions. Consequently penicillin injections were increased to 50,000 units intramuscularly every three hours during the fourth day of treatment. A remarkable improvement was noted during the following 19 hr., and this improvement continued until complete recovery. At intervals during convalescence the serum was tested with a flagellar and somatic antigen. An antibody titer of 1–160 with the "H" antigen and 1–80 with the "O" antigen was obtained on the 12th day following injection with the organisms, with the maximal titers (1–5120 with the "H" antigen and 1–1280 with the "O" antigen) being obtained on the 27th day following injection. Following a period of 190 days after the initial injection an unsuccessful attempt was made to reinfect the rabbit using 1,000,000,000 living organisms. Two weeks later the rabbit was bled to death and autopsied. Sections of the organs revealed only slight monocytosis of the spleen and liver. Grossly, the organs were not remarkable, and no evidence of permanent organic damage due to the infection was observed.

Pathogenicity in the Chick Embryo

Intra-allantoic inoculation of 10-day-old-chick embryos with 0.1 ml. of an 18 hr. broth culture serially diluted from 10,000 to 1,000 living organisms produced death of the embryo in 24 to 48 hours. Upon dissection, positive smears and cultures were obtained from the allantoic, amniotic, brain, and yolk fluid. There were poxlike plaques covering the chorioallantoic membrane surrounding the area of injection.

TABLE I

RESPONSE TO ONE RABBIT THAT WAS INJECTED WITH *Listeria monocytogenes* INTRAVENOUSLY AND TREATED WITH PENICILLIN

Time of count	Total leucocytes*	Polymorphonuclear cells*		Lymphocytes*		Monocytes*		Hemoglobin, gm.	Temperature†
		%	Absolute	%	Absolute	%	Absolute		
Normal rabbit‡	8866	51.33	4552	40.54	3595	8.10	719	10.5	104
Before injection	8000	57	4560	42	3360	1	80	10.5	104
After 48 hr.	9900	13	1287	82	8118	5	495	9.5	105.8
After 72 hr.	17600	23	3860	68	12056	9	1584	9.5	107
After 96 hr.	7850	26	2041	67	5259	7	549	9.0	108
After 120 hr.	13050§	34	4437	56	7308	10	1305	8.5	108
After 144 hr.	8050	61	4910	37	2979	2	161	10.0	105
After 168 hr.	21900‖	48	10512	42	9198	10	2190	10.0	105
After 216 hr.	26000	41	10660	41	10660	18	4680	10.0	105
After 240 hr.	13000	42	5460	43	5590	15	1950	10.0	102
After 264 hr.	19050#	46	8763	53	10096	1	190	10.0	103

* *Per cubic millimeter.*
† *Rectally.*
‡ *Blood count of 62 normal rabbits (Downey, Handbook of hematology, p. 1837, Vol. 3).*
§ *Penicillin therapy started.*
‖ *Relapse.*
\# *Asymptomatic.*

Pathogenicity in White Mice

A small number of living organisms injected either intraperitoneally or intracerebrally into mice produced death within 24 to 48 hr. Mice were used for oral administration of the organism to ascertain the possibility of oral infection. In these tests, mice weighing approximately 17 grams each were placed in an isolated cage and given water polluted with *L. monocytogenes* for a period of two days. Deaths were recorded daily. Of the 20 mice that were used, 16 had succumbed by the 12th day and the remainder survived the one month observation period.

Pathogenicity for Guinea Pigs

Guinea pigs were injected intraperitoneally with 1 ml. of living organisms ranging from 10,000 to 100,000 per ml. in number. In the 10 guinea pigs injected with 10,000 organisms per ml. and in 10 guinea pigs injected with 100,000 organisms per ml. death occurred in four to seven days. All showed signs of weakness, anorexia, dyspnea, and ruffling of the hair. On postmortem examination the liver was always necrotic. Occasionally there was central nervous system involvement.

The use of vaccine as a prophylactic measure in prevention and control of outbreaks of the disease in animals proved successful in studies done using guinea pigs. Guinea pigs weighing 250 gm. received intraperitoneal injections of a heat-killed antigen in five increasing doses, at intervals of four days. These animals were protected against an injection of 200,000 living organisms per ml. Control guinea pigs were injected at the same time with 200,000 living organisms per ml. with death following the typical course. Two weeks later, the surviving immunized guinea pigs were challenged with 1,000,000,000 living organisms per ml. whose virulence had been established by three mouse passages. Again, no illness occurred, but the control guinea pigs died with typical symptoms.

Attempts made to demonstrate toxin production by using a sterile filtrate of an 18 hr., 72 hr., and a 30-day-old broth culture were unsuccessful. Varying amounts of this filtrate were injected into rabbits, guinea pigs, and mice by the intravenous, intraperitoneal, and intracerebral routes without apparent illness. The results of the two strains tested were similar to the results obtained by Burn (2).

Sensitivity Studies

In determining the sensitivity of the *L. monocytogenes* strains to penicillin, the serial dilution technic* was used, mainly because these two strains differed from other reported strains by showing a higher degree of inhibition by penicillin. Foley, Epstein, and Lee (3) described seven strains of *L. monocytogenes* that grew freely in 40 times the concentration of penicillin which would have inhibited most other Gram positive organisms that they tested.

* *A method for determining the sensitivity of an organism to antibiotics Food & Drug Administration, Division of Penicillin Control & Immuniology, Washington 25, D.C.*

Hendelman *et al.* (7) reported one case extensively treated with penicillin and sulphadiazine, in which recovery was attributed to the latter drug. In the two strains tested in this research, the strain isolated in 1949 was found to be sensitive in a range between 0.09 and 0.18 units of penicillin per ml., and the strain of 1951 was sensitive to between 1.50 and 3.12 units of penicillin per ml.

In determining the effectiveness of other antibiotics against these two strains, the plate disc method was found to be satisfactory and the results are shown in Table II.

TABLE II

THE EFFECTIVENESS OF ANTIBIOTICS AGAINST *Listeria monocytogenes* USING THE SATURATED FILTER DISC METHOD IN THREE CONCENTRATIONS ON BLOOD AGAR PLATES

Antibiotic	1949 strain	1951 strain
Penicillin	+++	++
Streptomycin	++	+
Sulphathiazole	−	−
Sulphadiazine	−	−
Aerosporin	−	−
Bacitracin	++	++
Aureomycin	+++	−
Terramycin	+++	++
Dihydrostreptomycin	++	+
Chloromycetin	+++	+++

− = Not sensitive, + = slightly sensitive, ++ = moderately sensitive, +++ = highly sensitive. Read after 18 hr. incubation at 37° C.

Serological Tests

At intervals during the disease and convalescence, a bacterial antigen of *L. monocytogenes* was tested with sera obtained from the patient infected with the 1951 strain. There was no detectable antibody response five days after the onset of illness. Serum taken 42 days later gave a titer of 1–80 with both the "H" and the "O" antigen. A blood culture taken at this time was positive for the *Listeria* organism. Since the patient had been transferred, it was impossible to obtain sera between the 5th and the 42nd day. But sera were obtained by follow-up on the 71st and 78th day following the onset of illness with neither an antibody titer nor a positive blood culture being demonstrated.

Sera taken from rabbit No. 200 previously injected with *L. monocytogenes* were tested periodically to show a rise in antibody titer of the rabbit. The results of these serum agglutination tests during convalescence and post infection are shown in Fig. 1.

Immune serum taken 20 days after infection of rabbit No. 200 with the 1951 strain was used to demonstrate cross-agglutination between the 1949 and 1951 strains. Using "H" antigen these strains gave titers of 1–1280 and 1–2560 respectively, with "O" antigen the titers were found to be 1–320 and 1–1280 respectively indicating that these two strains of *Listeria* are related serologically.

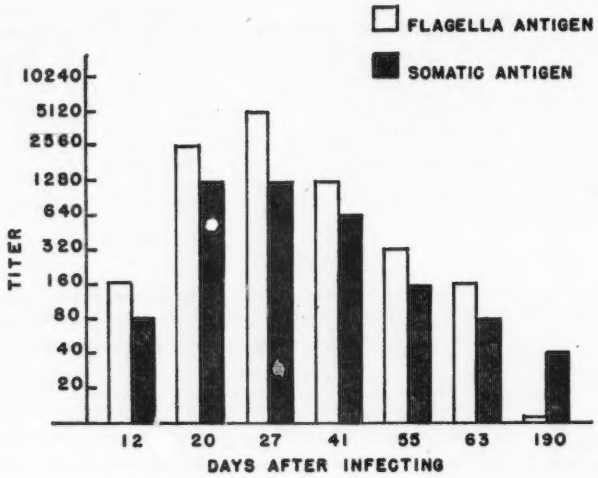

FIG. 1. Antibody responses after infection with *L. monocytogenes.*

Heat-labile Surface Antigen

Both strains were found to contain a comparable amount of a surface antigen which was demonstrated by absorption tests with the *E. coli* K8 antisera which is an "L" antigen with no somatic antigen relationship. The "L" antigen was found to reach its highest titer in cultures grown at 37° C. in 18 to 24 hr. Antigens prepared from broth cultures at 24-hr. intervals showed progressively decreasing titers and at 240 hr. no titer could be demonstrated. Cultures grown at 2.8° C. to 5° C. maintained the "L" antigen titer for at least six months without loss. The virulence of the organism is apparently correlated with the presence of this antigen (as the "Vi" antigen is related to virulence of *Salmonella*) as evidenced by the fact that mouse injections of the living organisms with no "L" titer did not produce death. Cultures grown at 37° C. over extended periods showed a loss in virulence with the age of the culture. Cultures (after growth at 2.8° C. to 5° C. over extended periods) when injected into mice showed a high degree of pathogenicity.

The antigenic material which agglutinates with the *E. coli* K8 antisera is apparently destroyed by removal from the cell body with chloroform. Living antigens that were agglutinated by a 1–2560 dilution of the K8 antisera, when washed with chloroform and retested, gave a titer of 1–20. After washed organisms were prepared as flagellar and somatic antigens, no loss in "H" or "O" titers could be demonstrated. Extractions with chloroform followed by three extractions with ether yielded a milky material which was found to react with the *E. coli* K8 antisera. When equal parts of antisera and the extract were mixed in a capillary pipette an immediate precipitation was observed. Negative reactions resulted from normal rabbit sera, normal

human sera, and sera taken from rabbits after infection with the *Listeria* organism. This extracted material (which is similar to the lipoid substance extracted from *L. monocytogenes* by Stanley (12) and designated as a monocytic producing agent (MPA) when injected intravenously into rabbits) does not produce any clinically discernible signs of illness. It does, however, give rise to a monocytosis. Further investigation on this material, its relationship to the heat-labile "L" antigen, and its possible role as a surface antigen important for virulence is in progress.

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THE USE OF BACTERIOPHAGE OF *XANTHOMONAS PHASEOLI* IN DETECTING INFECTION IN BEANS, WITH OBSERVATIONS ON ITS GROWTH AND MORPHOLOGY[1]

By H. Katznelson[2], M. D. Sutton[2], and S. T. Bayley[3]

Abstract

A study was made of certain factors affecting the use of a phage–plaque-count technique for detecting in bean seed the organism causing common bacterial blight, *Xanthomonas phaseoli* (Erw. Smith) Dowson. It was found that overgrowth of the fluid test cultures with contaminating bacteria from the seed or from soil did not interfere appreciably with phage adsorption or multiplication in the presence of host cells. Such samples required centrifugation prior to testing in order that plaques might be readily discernible on agar plates. The phage was adsorbed specifically only by cells of *X. phaseoli*. Tests with a variety of media showed that nutrient broth was adequate for the purposes of the phage–plaque-count technique. An incubation time of at least 24 hr. for phage and sample was shown to be desirable. Out of 75 samples tested in quadruplicate, 50% were found to be infected with the pathogen in varying degrees. Several samples gave positive reactions by means of this plaque-count method under conditions which were quite unfavorable for isolation of the pathogen by the usual methods.

One-step growth studies with *X. phaseoli*-phage indicated a low order of adsorption, a latent period of 30 to 45 min., a rise period of approximately 30 min., and a burst size of 40 for stationary cultures and 30 for shake cultures. Electron microscopy of *X. phaseoli*-phage preparations revealed a particle with a dense head 60 mμ in diameter and a tail 170 to 190 mμ long and 10 to 15 mμ wide.

Introduction

In previous publications (5, 6, 7) it was shown that it is possible to detect bacterial plant pathogens borne internally in seed by means of a specific phage–plaque-count technique. It was demonstrated that this method could be applied to the detecting of the halo blight organism, *Pseudomonas phaseolicola* (Burk.) Dowson, in bean seed, bacterial blight of peas caused by *P. pisi* Sackett, and probably also common blight of beans caused by *Xanthomonas phaseoli* (Erw. Smith) Dowson. The method was more rapid, simpler, and more effective than other commonly used procedures which entail the isolation of the organism and the confirmation of its identity by pathogenicity tests, greenhouse or field trials, or the use of serological methods, for the determination of infection in seed. Further data on the application of this plaque-count procedure to the detection of *X. phaseoli* in beans have been accumulated during the past two years and certain modifications of the technique have been made. In addition a study has been carried out on the growth of *X. phaseoli*-phage and on the morphology of the phage particle itself.

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[3] *National Research Council.*

Procedure

The details of the phage method for detecting seed-borne infection have been published elsewhere (5) but will be outlined again in order that the several changes and additions may be recorded. The number of bean seeds per sample tested at one time was changed from 50 to 75 and the volume of the suspending nutrient broth from 250 to 300 ml. Each seed sample was first surface sterilized for 10 min. in a bleaching powder solution standardized to 2% free chlorine, washed in several changes of sterile water, and ground aseptically for five minutes in sterile nutrient broth in a Waring blendor. These ground samples were held at room temperature overnight, shaken thoroughly, and after the coarse particles had settled, 10 ml. of each ground sample was transferred to sterile 50 ml. Erlenmeyer flasks containing 0.1 to 0.5 ml. of a suitable phage dilution to provide 4,000 to 8,000 phage particles per flask. Immediately 0.1 ml. aliquots of this mixture were removed from each flask to Petri plates containing 0.5 ml. of a nutrient broth suspension of *X. phaseoli* from a 24-hr. yeast beef agar slant. At the same time, 0.1 ml. portions of the incubated ground samples were also plated in a similar manner to determine whether the bean samples harbored latent phages of *X. phaseoli*. Ten milliliters of yeast beef agar was poured into each plate. Suitable controls of nutrient broth alone and with phage were also plated with the host cell suspension. After 16 to 18 hr. incubation at room temperature, the 10-ml. samples of the ground samples containing phage were centrifuged aseptically and 0.1 ml. aliquots of the supernatant fluids plated for plaques. These were usually visible within 6 to 10 hr. A significant increase in the number of plaques over the number added to each flask was taken as indication of the presence in the sample of the susceptible host cells and therefore of infection of the beans with the common blight organism.

Some Factors Affecting Use of the Plaque-count Procedure

Overgrowth of plates with a variety of organisms, other than the pathogen, which may be casual contaminants or from within the seed and therefore not eliminated by surface sterilization, was frequently a serious hindrance. In such cases plaques were often completely obscured. This was corrected by centrifuging the 10-ml. samples in sterile tubes and plating 0.1 ml. aliquots from the supernatant fluid. Striking results were obtained by this slight modification as may be seen in Figs. 2 and 3. Tests were made on the effect of large numbers of a variety of organisms in soil on the phage count, by adding a known number of phage particles to a suspension of soil in nutrient broth and making plaque counts periodically. In a similar test, phage was added to the soil suspension after the latter had been incubated overnight and was heavily overgrown with bacteria. Under these conditions centrifugation of all samples was necessary. It was observed (Table I) that there was no significant decrease in plaque-count as a result of adding large numbers of soil microorganisms. When *X. phaseoli* was added to the above mixtures there was a striking increase in the number of phage particles, indicating that

TABLE I

EFFECT OF SOIL MICROBIAL POPULATION ON *Xanthomonas phaseoli*-PHAGE

Sampling period (hours)	Treatment of sample	Number of plaques per plate			
		Phage added at once to soil suspension		Phage added after soil suspension incubated overnight	
		X. phaseoli added	*X. phaseoli* not added	*X. phaseoli* added	*X. phaseoli* not added
0	Centrifuged	48	45	50	44
	Not centrifuged	46	52	Obscured	Obscured
5	Centrifuged	C	46	C	56
	Not centrifuged	C	48	Obscured	Obscured
24	Centrifuged	C	35	—	—
	Not centrifuged	Obscured	Obscured	—	—

C = too many plaques to count, or confluent.

the contaminating organisms did not interfere appreciably with phage multiplication on its specific host. That *X. phaseoli* specifically adsorbs its phage was demonstrated by comparing the degree of adsorption of phage by different strains of *X. phaseoli*, one strain each of the closely related *X. phaseoli* var. *fuscans* (Burk.) Starr and Burkholder and var. *sojensis* (Hedges) Starr and Burkholder and several unrelated organisms isolated from beans. The experiment was carried out by mixing, in sterile nutrient broth, a known number of phage particles with a considerable excess of young bacterial cells from each of the above cultures and plating for plaques at once and after 15 min. at which time each of the samples was centrifuged to throw down the adsorbed phage and cells and the supernatant fluid plated. There was a 60% and 77.5% reduction in number of plaques by strains *X. phaseoli*—Xp 8 and RT 16-4, respectively, but no adsorption by the closely related *X. phaseoli* var. *fuscans* and var. *sojensis* species or by unrelated yellow saprophytic bacteria.

In a previous study it was shown that bean tissue itself could inhibit or adsorb *P. phaseolicola*-phage but that not all bean samples had this property. Similar experiments for *X. phaseoli*-phage were set up with three common bean varieties ground for 5 and for 10 min., with and without added *X. phaseoli* cells. It was found that no phage adsorption of any consequence occurred nor was there any apparent influence on phage multiplication in the presence of the host cells. However, in the course of analyzing a considerable number of bean samples, several were encountered which appeared to reduce the plaque count by as much as 50 to 75% but did not appear to interfere with phage multiplication if the host cells were present.

A number of attempts were made to improve or render more specific the ground bean sample as a medium for multiplication of both *X. phaseoli* and

its phage. To this end, yeast extract was added to the nutrient broth, a bean extract with glucose and yeast extract was substituted for nutrient broth, and nutrient broth was buffered with phosphate or with calcium carbonate. In no instance was there evidence of improvement over nutrient broth alone. In fact the addition of calcium carbonate interfered with phage multiplication. The addition of yeast extract also reduced markedly the plaque count possibly by stimulating a very profuse overgrowth of contaminating bacteria.

Size of sample was also an important consideration. Originally a total of 200 bean seeds was used, later this was increased to 300, which were most conveniently ground in 4 lots of 75 each. Most of the samples which showed no increase in plaque count, and were, therefore, presumably free from *X. phaseoli*, again failed to do this when fresh aliquots were tested (Sample X39, Table II). One sample (X40, Table II) showed an increase in number of plaques when retested. This increase indicated very light infection as compared with samples X51 and X59 (Table II) which were heavily infected. The testing of 300 seeds per sample was an arbitrary choice dictated by the equipment available. Any number of seeds may be tested although the grinding of more than 75 seeds in less than 300 ml. broth resulted in a very thick mixture which caused complications with regard to sampling and adsorption or inactivation of phage. It was also felt that too deep a layer in the Mason jars used to prepare the samples was undesirable as *X. phaseoli* is strongly aerobic.

TABLE II

DEGREE OF INFECTION OF DIFFERENT SAMPLES OF BEANS WITH *X. phaseoli* AS DETERMINED BY THE PLAQUE-COUNT METHOD

Bean Sample No.	Plaques per plate from replicates (75 seeds each)			
	1	2	3	4
X39 A	75	63	70	66
B	56	40	66	60
X40 A	70	50	75	65
B	64	56	C	60
X51	C	200	C	C
X55	60	C	75	76
X59	C	C	51	C
X62	350	56	55	66
X72	220	C	45	55

C = too many plaques to count, or confluent.

Originally, a six-hour incubation period of the ground samples containing phage appeared to be adequate for *P. phaseolicola*-phage when used to detect halo blight in bean seed (5). With *X. phaseoli*, however, it was noted that a longer incubation period was required to build up a significantly higher virus count in certain infected seed samples (Table III). Consequently samples were incubated with phage for 18 to 24 hr. before plaque counts were made.

TABLE III

EFFECT OF TIME OF INCUBATION OF BEAN SAMPLE WITH PHAGE ON PLAQUE COUNT

Sample No.	Number of plaques per plate at		
	0 hr.	6 hr.	24 hr.
X26–1	89	86	86
–2	61	C	C
X28–1	70	8	C
–2	96	78	512
X29–1	102	80	556
–2	85	75	600
X30–1	60	60	57
–2	72	536	C
X31–1	70	C	C
–2	72	8	C

C = too many plaques to count, or confluent

Results of Tests

In all, 75 samples of bean seed were tested in quadruplicate (75 seeds × 4 = 300 seeds per sample). Approximately 50% of these samples were found to harbor the common blight organism. As has been illustrated in Table II, some of these samples were heavily infected (X51, X59), others only very slightly (X39, X40). Confirmation of the presence of *X. phaseoli* was achieved during the early work (samples XI–X25) by plating every ground sample and making isolations of yellow pigment-producing organisms. These isolates were all exposed to phage in nutrient broth and inoculated into young bean seedlings to determine their pathogenicity, as was done with halo blight of beans (5) and bacterial blight of peas (7). In no instance was *X. phaseoli* isolated from samples which were negative by the plaque-count technique. Samples showing the presence of common blight by this technique, with one or two exceptions, yielded *X. phaseoli* cultures which were lysed by its phage in broth and which were pathogenic to bean plants. The instances in which positive samples did not yield *X. phaseoli* on plating occurred with samples which were lightly infected and in which overgrowth with contaminating bacteria was possibly heavy enough to prevent the

development or recognition of *X. phaseoli* on the agar plates. This observation suggests that it may be possible by the phage procedure to detect seed-borne infection under conditions which are quite unfavorable for demonstrating the presence of the pathogen by other methods.

One-step Growth Studies and Electron Microscopy

A number of phages for phytopathogenic bacteria have been isolated but their adsorption and multiplication characteristics have not been studied extensively. Chapman *et al.* (1) have done this with a phage for *Erwinia carotovora*.

In the present work, 12-hr. glucose nutrient broth shake cultures were used, the final mixture containing a ratio of phage particles to bacteria of 1 : 2.5. Both shaken and stationary cultures were tested. *X. phaseoli*-phage was adsorbed slowly, consequently a contact period of 25 min. was allowed before samples were taken for dilution, to prevent further adsorption, and for centrifugation to determine the degree of adsorption according to the method described by Delbrück and Luria (2). The diluted series were sampled at 15-min. intervals to determine the latent and rise periods and the burst

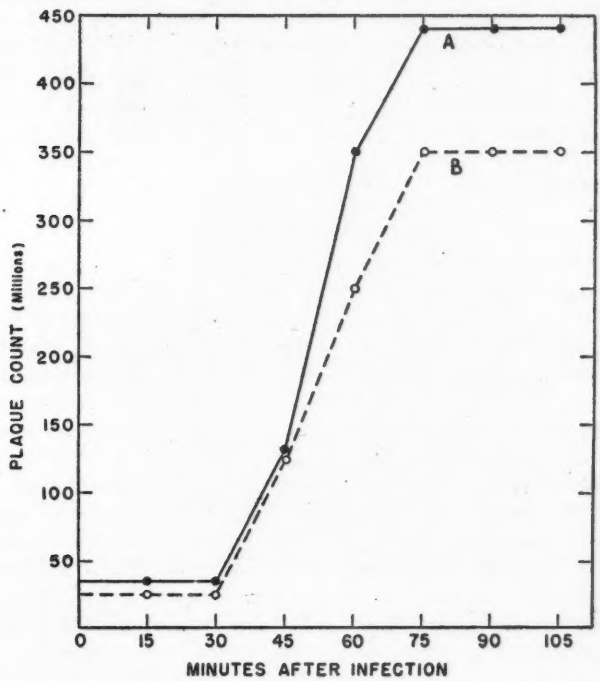


FIG. 1. One-step multiplication of *Xanthomonas phaseoli*-phage at 22°C. in nutrient broth. (*A*) Stationary. (*B*) Shaken.

size. Adsorption amounted to 20% at 25 min. and, as may be seen in Fig. 1, the latent period was between 30 and 45 min., the rise period was approximately 30 min. The burst size was found to be 40 for stationary cultures and 30 for shake cultures.

In general the morphology of phages for plant pathogenic bacteria (1, 4) is similar to that of many other phages (3, 8, 11). *X. phaseoli*-phage was partially purified by successive centrifugations at high and low speeds in a Spinco preparative centrifuge as suggested by Williams and Fraser (8), with the final residue being suspended in distilled water. Drops of this preparation were placed on specimen grids and shadowed with chromium.

As shown in the electron micrographs Figs. 4 to 6, the phage particles appear to have dense heads about 60 mμ in diameter and tails 170 to 190 mμ in length and 10 to 15 mμ in width. Thus this phage also conforms in general spermlike morphology to those described by other workers. There is evidence in some micrographs (Fig. 5) that the heads are actually many sided. Some damage to the phage particles may have resulted during preparation since ghost particles are found (Fig. 6). The relationship of the phage to its host bacterium was investigated by making collodion pseudoreplicas of suitable agar plates (11), mounting the replicas on specimen grids and shadowing with chromium. Phage particles were found adsorbed on the periphery of the cell; their orientation suggested an attachment by their tails which may have penetrated the cell, although the evidence for this was by no means conclusive. Certain preparations (Fig. 7) showed filaments which appear to consist of spheres about the size of the virus heads as observed also by Wyckoff (9, 10) with several T phages. Fig. 7 also shows close packing of individual phage heads.

Discussion

It is clear that a phage as active and specific as that for *X. phaseoli* makes it possible to detect the common blight organism in bean seed in the laboratory both rapidly and simply. This applies also to the diagnosis of halo blight of beans with *P. phaseolicola*-phage (5). The isolation of phages for the *X. translucens* group of organisms (6) also suggests a possible application of the phage–plaque method to the determination of bacterial infection of cereal seed. However, the use of this method need not be restricted to seed. In the light of the results obtained with *X. phaseoli* in soil (Table I) it is possible that soil-borne bacterial pathogens of plants can be detected in soil with species specific phages. In general, it would appear that the most useful application of this method lies in its ability to detect specific organisms in an environment or under conditions which are particularly unfavorable for their demonstration or isolation by the conventional techniques of plating, plant inoculation, plant growth, etc. For this purpose species specific phages are essential.

Acknowledgment

The authors are indebted to Mr. W. Bell for technical assistance.

PLATE I

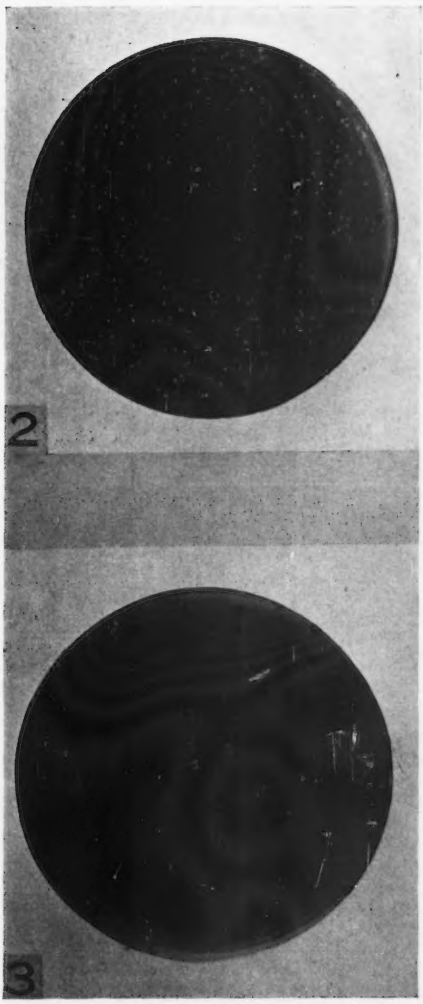
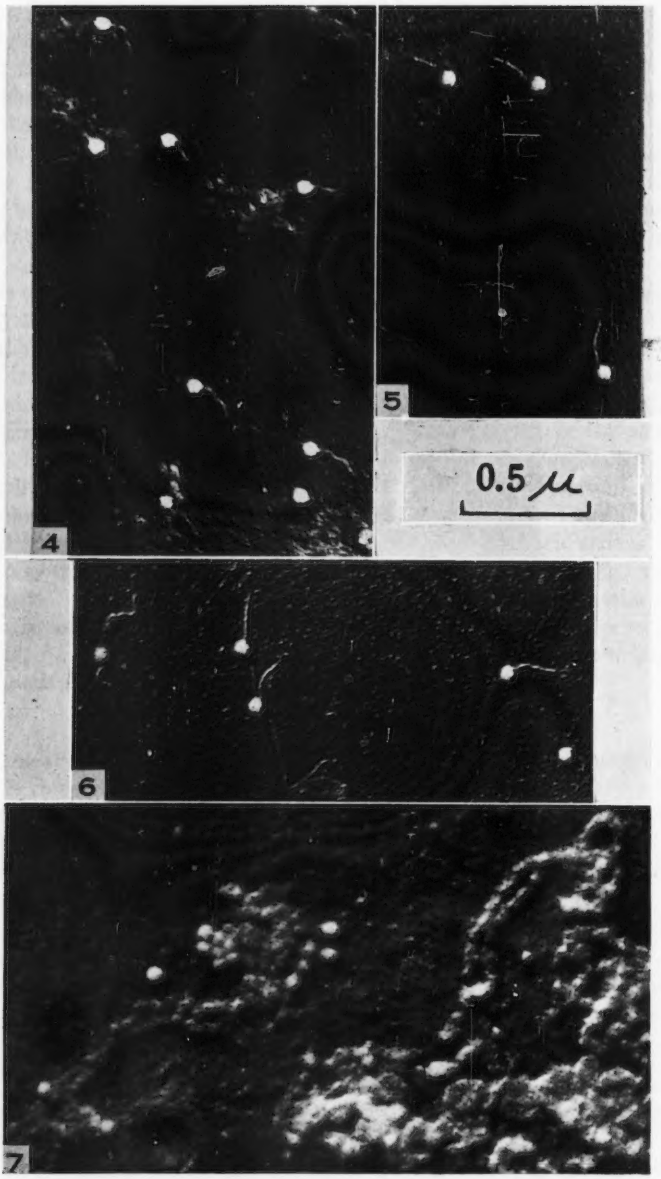


FIG. 2. Influence of centrifugation of ground bean sample on visibility of plaques of *Xanthomonas phaseoli*-phage. Sample not centrifuged, plated directly.

FIG. 3. Influence of centrifugation of ground bean sample on visibility of phage-plaques. Sample centrifuged, supernatant fluid plated.

PLATE II



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FIGS. 4–7. Electron micrographs of partially purified *Xanthomonas phaseoli*-phage and of the residue of a lysed cell. Chromium shadowed. FIGS. 4 to 6. Showing sperm-like morphology of phage particles with a suggestion that the heads are many sided. Fig. 6. Showing "ghost" particles. FIG. 7. Showing beaded filaments and close packing of viruslike bodies in the residue of a lysed cell.

SLIDE CULTURE OF TUBERCLE BACILLI

II. *IN VITRO* SENSITIVITY TESTING[1]

By R. W. Reed[2]

Abstract

A technically simple method of testing the sensitivity of tubercle bacilli to tuberculostatic agents is described which gives accurate results directly from positive sputum in one or two weeks. Parallel slide and tube sensitivity tests against streptomycin and isoniazid on 136 specimens show 49% and 68% agreement respectively. The number of cultures within the sensitive range of each drug was almost identical. The advantages of slide sensitivity testing over conventional methods are technical simplicity and rapid results.

Introduction

It is generally agreed that there is an urgent need for a simple, rapid, and reliable method of testing the sensitivity of *Mycobacterium tuberculosis* to various tuberculostatic agents. Present techniques are slow and often cumbersome and the results are frequently delayed to the point where they are of no more than academic interest to the clinician. Most conventional methods used for determining sensitivity are subject to the criticism of delay. The commonest method involves preliminary isolation on Lowenstein's or equivalent medium, followed by testing in a fluid medium (17), and requires six to eight or more weeks. Another common method employs direct culture of digested sputum on solid media containing appropriate concentrations of the agent to be tested. Karlson and Needham (13) and Youmans *et al.* (25) have used egg yolk agar media extensively, while Anderson (1) has used potato–egg medium. Results are rarely readable in less than three weeks and usually require longer.

The results of sensitivity tests should be available to the clinician within one or two weeks of taking the specimen to be of use as a guide to future therapy. A method is currently available which fulfills this need, although it has been accorded little attention in this country. The successful use of slide cultures for sensitivity testing of *M. tuberculosis* has been reported from France (3 to 8, 18), Chile (24), Australia (21), England (9, 20, 22), and the United States (10, 12). Several media have been used with varying results including saponated or lysed blood media (12, 20, 22), Dubos' medium with (10) and without Tween 80 (9), and a serum-synthetic medium (21). Some of the media and procedures above were much too complicated for routine use, although found to be satisfactory by their originators. This report is an extension of the author's earlier work with a rapid slide culture method (19) which has been adapted not only for routine diagnostic use (23) but also for routine sensitivity testing of *M. tuberculosis* against streptomycin and isoniazid.

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Contribution from the Department of Bacteriology, Dalhousie University, Halifax, and the Division of Laboratories, Department of Public Health, Nova Scotia.
[2] *Professor of Bacteriology, Dalhousie University and Provincial Bacteriologist.*

Methods

The procedure used throughout is that described in the previous paper (19). In brief, sputum was homogenized and thick smears on standard slides were dried for one hour at 37° C., decontaminated in 6% sulphuric acid, and flushed three times with sterile distilled water. Slides were then immersed in Kirchner's medium (14) enriched with 10% pooled sterile human serum plus 1 : 1,000,000 gentian violet (15) and incubated at 37° C. Decontamination tubes and horizontal culture tubes have been described by Lowry and Berry (16). The details of the laboratory setup will be described elsewhere (23).

Streptomycin was added to the serum-synthetic medium to give final concentrations of 0.1, 1.0, 10.0, and 100 μgm./ml. Concentrations of isoniazid used were 0.01, 0.1, 1.0, and 10.0 μgm./ml. Ten slides in all were prepared from each specimen. Four were incubated in serum-synthetic medium plus streptomycin, four in medium plus isoniazid, and two in tubes of drug-free medium to serve as controls. After incubation for seven days, one control slide was removed, fixed, stained with Aubert's cold stain (2), and examined under the low power. If satisfactory growth had occurred, all slides were examined. If not, the remaining slides were incubated for another week before the test was read. The slide sensitivity end point was taken as the lowest concentration of drug which showed complete or marked inhibition of growth and cord formation as compared with the control slide.

After smears had been prepared from the homogenized sputum for slide sensitivity testing, the sputum was concentrated and inoculated to tubes of Herrold's egg-yolk agar. Concentration was accomplished by incubation of sputum with an equal volume of 20% trisodium phosphate at 37° C. for 24 hr. This was followed by neutralization with 10% hydrochloric acid in the presence of 1% bromthymol blue and centrifugation at 3000 r.p.m. for 30 min. When growth became apparent, the entire growth was emulsified in sterile normal saline and a small amount inoculated to Dubos' Tween-albumin medium (11). When growth reached the density of MacFarland Tube No. 1 (3×10^8/ml.), 0.1 ml. was inoculated to tubes of Dubos' medium containing concentrations of streptomycin or isoniazid noted above. The end point was read as the lowest concentration showing complete inhibition of growth.

Slide sensitivity tests were also performed on tubercle bacilli first isolated on solid media. In these instances, drug-free Dubos' medium was inoculated as above. When sufficient growth had appeared, the Dubos' culture was diluted 1 to 10 in sterile human serum and slides were prepared from this.

Results

Preliminary experiments demonstrated that the end point of slide sensitivity tests was very difficult to evaluate unless fairly heavy growth was present on control slides for comparison. Accordingly only sputum which was positive on direct smear was used in order to ensure good growth. We were able to

obtain 136 such specimens from patients in tuberculosis hospitals. Streptomycin and isoniazid sensitivity tests were done by slide culture and tube dilution methods described above.

The results of streptomycin tests are shown in Table I. There was agreement between the two methods of testing in 67 of 136 specimens (49%), as shown in heavy type. The tube test tended to give a lower end point than the slide test, particularly at 0.1 μgm./ml. At this concentration, 62 were inhibited in the tube test and 40 in the slide test. The reverse is true at a concentration of 1.0 μgm./ml. A total of 98 specimens showed inhibition by 1.0 μgm./ml. or less by the slide method and 100 by the tube method. Of these 55 were in agreement. It was observed that in many cases where the tube test showed complete inhibition (judged by naked eye examination) the slide test showed the presence of a few large serpentine colonies at the same concentration but few or no cords at the next highest concentration. This presumably indicates the presence of some resistant mutants in the population under test.

TABLE I

STREPTOMYCIN SENSITIVITY OF *M. tuberculosis:* COMPARISON OF SLIDE AND TUBE TESTS ON FRESHLY ISOLATED STRAINS

(Heavy type—agreement of the two methods)

Inhibiting concentration of streptomycin in μgm./ml.

Slide test \ Tube test	0.1	1.0	10	100	> 100	Totals
0.1	**30**	5	2	1	—	40
1.0	24	**25**	3	3	3	58
10	5	7	**5**	1	1	19
100	—	—	3	**3**	4	10
> 100	3	1	—	3	**4**	11
Totals	62	38	13	11	12	136

Isoniazid sensitivity tests done by the two methods are summarized in Table II. In this case there was agreement in 92 of 136 specimens or 68%. The slide test in general gave lower end points than the tube test. In the majority of cases, there was complete inhibition of growth, so that isoniazid slide end points were much easier to read than streptomycin slide end points. A total of 92 specimens were inhibited by 0.1 μgm./ml. or less on slide test as compared with 101 by tube test. The appearance of resistant mutants was less frequent than in streptomycin slide tests. A small number of tests were done by the two methods using *p*-aminosalicylic acid and in most cases there was complete agreement.

TABLE II

ISONIAZID SENSITIVITY OF *M. tuberculosis:* COMPARISON OF SLIDE AND TUBE TESTS ON FRESHLY ISOLATED STRAINS

(Heavy type—agreement of the two methods)

Inhibiting concentration of isoniazid in μgm./ml.

Slide test \ Tube test	0.01	0.1	1.0	10	> 10	Totals
0.01	**30**	—	3	—	1	34
0.1	9	**45**	7	4	2	67
1.0	—	6	**6**	1	—	13
10	—	1	6	**7**	1	15
> 10	—	1	—	2	**4**	7
Totals	39	53	22	14	8	136

At present, slide sensitivity tests are being done routinely on a number of patients in the Halifax Tuberculosis Hospital either directly from positive sputum or from cultures first isolated on solid media where sputum is negative on smear. Cultures from any single patient show consistent degrees of sensitivity or resistance to streptomycin and isoniazid on specimens taken over short time intervals. It is obvious that in some cases resistance to one or both agents is developing during treatment.

Discussion

The management of individual cases of tuberculosis must be based on clinical, radiological, and bacteriological evidence. Determination of sensitivity to tuberculostatic agents should form an important part of this evidence, since it is now well established that strains of tubercle bacilli resistant to streptomycin or isoniazid emerge during treatment. Owing to the time required for the performance of conventional sensitivity tests, the results are of little or no use as a guide to therapy.

The slide sensitivity test in our opinion satisfies both the clinical and laboratory requirements of such a test. For the clinician accurate results are available in one to two weeks after the test is requested. For the laboratory, the test is technically simple, utilizing an easily and quickly prepared medium, and requires only very small amounts of sputum. Tedious preparation of solid media and time-consuming digestion and concentration of sputum are avoided. Contamination is rarely encountered.

Slide culture has been demonstrated to be more sensitive than conventional culture methods (19, 23). Rubbo and Morris (21) compared slide and tube tests quantitatively using mixtures of streptomycin sensitive and resistant tubercle bacilli with entirely satisfactory results. All workers have found that direct slide sensitivity tests can be done only when sputum is positive on direct smear. This limitation, however, is not a serious one since the cases requiring detailed investigation of sensitivity are usually those with positive sputum. If sputum is positive on culture but negative on smear, the slide test can still be performed on tubercle bacilli isolated on conventional solid media with some saving in time over tube tests. A test utilizing solid media with tuberculostatic agents incorporated can also be used on culture-positive, smear-negative specimens but in most cases such a test is subject to the same difficulty as slide tests, i.e. insufficient control growth for adequate comparison.

Direct slide tests and parallel tube tests have been done on 136 positive sputum specimens. In addition, slide tests alone against streptomycin, isoniazid, and PAS have been done on nearly 200 sputum specimens either directly or on cultures first isolated on solid media. The results of these tests have been satisfactory both technically and clinically. We are now using this method routinely for testing sensitivity of tubercle bacilli to streptomycin, isoniazid, and PAS. The method will also be useful for rapid testing of new tuberculostatic agents as they appear.

Acknowledgments

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EQUILIBRATION OF SUCCINATE SOLUTIONS WITH ADAPTED AND UNADAPTED AZOTOBACTER CELLS[1]

By Anna Maria Williams and P. W. Wilson

Abstract

Sucrose-grown (unadapted) and succinate-grown (adapted) cells were mixed with various concentrations of succinate at 3° C. under air and at 30° C. under helium. The amount of succinate removed from the external solution by the cells was proportional to the concentration of succinate and did not differ significantly with the two types of cells. Although the data did not differentiate between equilibration with intracellular water and mere adsorption, they indicate that no change has occurred in the surface of the adapted cells to allow a more rapid diffusion of succinate across the osmotic barrier under nonmetabolic conditions. So far as can be determined by using masses of cells, any "permeability" changes during adaptation of the azotobacter cells to succinate must be connected with an active transport process associated with metabolism.

Introduction

Although most work on biological cell permeability has been with the larger animal and plants cells (reviewed by Davson and Danielli (3)), an increasing number of investigations with bacteria have been made in recent years. Studies on penetration of dissolved substances into bacterial cells include those of: Yegian and Budd (14) on permeability to sulphonamides; Gale and co-workers (4, and succeeding papers in series) on assimilation of amino acids; Ørskov (8) and Cowie *et al.* (2) on permeability to sodium and potassium ions; Maas and Johnson (6) and Pollock (10) on penicillin uptake; and Mitchell (7) on phosphate transport. Conway and Downey (1) have investigated the penetration of succinic acid and other substances into yeast cells.

In addition to specific studies on penetration, the question of "permeability" often arises in discussions of microbial metabolism; "change in permeability" is a convenient explanation to cover a multitude of puzzling data. However, this expression may refer not only to diffusion across a cell membrane, but also to all factors (such as transport enzymes) necessary to carry the substrate across the osmotic barrier and into the enzyme cycle. Thus permeability in its broadest sense is not easily studied in microbial cells with the methods now available.

In manometric tests, sucrose-grown (unadapted) cells of *Azotobacter vinelandii* have a low initial rate of oxidation on succinate but increase during an adaptive or lag period of one to three hours to a high activity (13), whereas succinate-grown cells have a high initial rate of oxidation. However, cell-free extracts from both sucrose-grown and succinate-grown cells oxidize succinate, and the differences in activity are not sufficient to explain the large difference

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in initial activity of adapted and unadapted cells. It appeared worthwhile to test the simplest hypothesis of a change in permeability: whether adaptation includes a change in the inert cell surface which enables a faster diffusion of succinate into adapted cells under nonmetabolic conditions.

Methods

Growing and Harvesting of Cells

Azotobacter vinelandii O (ATCC 9104) was grown at 30° C. in 10-liter serum bottles containing 6 liters Burk's mineral salts medium plus 2% sucrose or 0.5% succinate. The bottles were aerated by passing sterile air through sintered glass spargers. The pH of the succinate medium was maintained near neutrality by periodically adding succinic acid during the growth period. Cells were harvested at 24 hr. in a Sharples centrifuge, washed in 10 volumes of 0.2% potassium chloride and recentrifuged, then suspended in 2 or 3 volumes of potassium chloride and centrifuged in an International refrigerated centrifuge for 15 min. at 23,000 × *g*. Cells thus treated contained 74–76% water.

Equilibration at 3° C. Under Air

After the cells had been weighed into a tared 25-ml. lusteroid centrifuge tube, a succinate solution buffered at pH 7.0 with 0.04 *M* phosphate buffer (in final volume, the buffer concentration approximated the concentration used in manometric experiments) was pipetted into the tube. The volume usually added was approximately equal to the volume of water in the cells, as determined by dry weights. The cell mass, after being rapidly stirred into a homogeneous suspension with a small glass rod and incubated for varying lengths of time, was then centrifuged at 23,000 × *g* for 15 min.; all subsequent centrifugations were carried out under these conditions. The supernate was poured off, and traces of liquid clinging to the cell mass were removed with filter paper. The cells were assayed either without washing or first given one or more washes with the 0.04 *M* buffer solution, the procedure for equilibration and centrifugation being the same as with the succinate solution.

Equilibration at 30° C. Under Air and Under Helium

Cells were weighed into a 150–ml. Warburg flask and shaken in a bath at 30° C., under air or under helium; succinate solution was tipped in from the sidearm. After the exposure period the flask (in closed-off system) was removed to the cold room and the cell suspension transferred to a centrifuge tube and treated as in the procedure for equilibration at 3° C. As the transfer was not entirely quantitative, the cells were reweighed after centrifugation, and assay values for buffer washes and cells were calculated for the original weight of cells.

Assay of Supernates and Buffer Washes

Succinoxidase was prepared from pig heart and used in a manometric assay for succinate as described by Umbreit, Burris, and Stauffer (12), except that

the succinate solutions were not extracted with ether. A 0.5 or 1.0 ml. aliquot of each solution (supernate or buffer wash) was assayed along with a corresponding aliquot of the standard succinate solution (10 μM./ml.–400 μM./ml.) used for equilibration. Concentrations higher than 80 μM./ml. were diluted to about 40 μM./ml. Duplicate flasks varied by not more than 4 μl., less than 2%. Corrections were made on each test solution by means of a blank run on the succinoxidase preparation and by the use of controls containing no succinoxidase.

Assay of Cells

Of the various methods tried for preparing cells to be assayed (such as boiling or autoclaving with and without acidification) the simplest and most satisfactory method was the following: The supernate (or last buffer wash if cells were washed before assay) was poured off and traces of liquid removed with filter paper, 4 ml. of 1 *N* hydrochloric acid was added, and the cell mass was made into a homogeneous suspension with a small glass rod. After standing about 30 min., the suspension was quantitatively transferred to a 25-ml. graduated cylinder, and made to volume, 12 to 20 ml., with 2 ml. of 2 *N* sodium hydroxide and distilled water washings.

Results

Equilibration of Low Concentration of Succinate at 3° and 30° C.

Initial experiments with succinate solutions of 40 μM./ml., a convenient concentration for the manometric assay, showed no significant difference in uptake of succinate between sucrose-grown and succinate-grown cells equilibrated at 3° C. The succinate not recovered in the supernate could be removed from the cells by equilibration with the phosphate buffer solution; two washes, each equal to the volume of succinate solution used for equilibration, gave a total recovery of 95–99%. In the experiment shown in Table I

TABLE I

EQUILIBRATION OF LOW CONCENTRATION OF SUCCINATE AT 3° C. AND 30° C.

5.0 gm. of cells; 5.0 ml. 40 μM./ml.	3° C. 10 min.*	30° C. 10 min.*
Sucrose-grown cells		
Supernate	15.0	14.6
5.0-ml. wash	3.3	1.9
Recovery in supernate	76.5%	74.5%
Total recovery	93.4%	84.2%
Succinate-grown cells		
Supernate	14.4	10.4
5.0-ml. wash	3.5	0.8
Recovery in supernate	73.5%	53.1%
Total recovery	91.3%	57.2%

**Assay values are reported in μM./0.5-ml. sample. Standard solution: 3°C., 19.6 μM.; 30°C., 19.6 μM.*

only one wash was given. At 30° C. under air where oxidation occurred, obviously more succinate would disappear from solutions equilibrated with the adapted cells; hence only a part of the succinate could be recovered.

When the mass of cells was washed free of succinate and again mixed with succinate solution, the same amount of compound was taken up; that is, previous exposure did not increase the affinity of the cells for succinate. In equilibrations of 1, 2, 3, and 4 gm. of cells, the amount of succinate remaining with the cells was proportional to their mass. When the volume of intracellular water was approximately equal to the volume of equilibrating solution, the percentage of the succinate taken up by the cell mass at 3° C. was considerably less than that expected had this compound been equally partitioned between extracellular and intracellular water. For example, from the data in Table I, 75% of 5.0 gm. = 3.8 gm. or ml. of water in this mass of cells, so if the 5.0 ml. of succinate solution equilibrated with the entire 3.8 ml., only 57% of the succinate should be recovered in the supernate. Instead, 76 and 73% remained in the external solution, indicating a volume of 1.6–1.8 ml. available for equilibration. However, this lack of equal partitioning was not too unexpected, since part of the intracellular water as determined by dry weights could be nonsolvent water. No significant difference was obtained between equilibration periods of 10 min. and 60 min. or between 30 min. and 120 min., so insufficient time of exposure was evidently not the cause.

Diffusion Versus Adsorption

Because of this unequal distribution, it was not known whether the succinate disappearing from the external solution was actually diffusing into the cell and equilibrating with the intracellular water, or merely being adsorbed to the surface of the cells. Maas and Johnson (6) studying penicillin uptake by *Micrococcus pyogenes* var. *aureus* over a wide range of concentrations (up to a 0.6% penicillin solution) pointed out that, if the uptake of penicillin by the cells were due to adsorption rather than to equilibration with intracellular water, some evidence of saturation would probably have occurred. In the experiments with succinate, the lowest concentration feasible to use with the manometric assay is about 10 μM./ml. and the highest without running into solubility difficulties is about 400 μM./ml., a 4.7% solution. Over this range no evidence of saturation was obtained; in fact, as the concentration of the equilibrating solution was increased, the percentage of succinate removed from the external solution increased slightly.

Equilibration of Sucrose at 3° C.

As another test of diffusion versus adsorption, equilibrations were carried out with 40 μM./ml. solutions of sucrose using the Folin-Wu method (5) for analyses. As determined manometrically, sucrose-grown cells oxidize sucrose immediately at a high rate of activity, whereas succinate-grown cells oxidize this compound at a very low rate which increases only slightly with time. Equilibration of sucrose with the two types of cells did not significantly differ,

and no change occurred between periods of 15 min. and 60 min. (Table II). The quantity of sucrose removed by the cells indicated an equilibrating volume of 0.9 ml. intracellular water, which is 41% of the 2.2 ml. calculated from dry weights. These results compare favorably with those for the succinate equilibration (Table I); with succinate the apparent equilibrating volumes were 42 and 47% of the volume calculated from dry weights. In some of the later experiments the apparent equilibrating volumes were higher, 50–75% of the dry weight volumes. However, small variations in the percentage recovery of substrate from supernate will cause striking differences in the calculation for apparent equilibrating volumes. This is especially true when the volume of equilibrating solution is much larger than the volume of cell water, such as in the experiment of Table V in which 2.5 gm. of cells were equilibrated with 5.0 ml. solution.

TABLE II

EQUILIBRATION OF SUCROSE SOLUTIONS AT 3° C.

2.9 gm. of cells; 3.0 ml. 40 μM./ml.	15 min. mgm./ml.	60 min. mgm./ml.
Sucrose-grown cells		
Supernate	9.78	10.22
3.0-ml. wash	1.42	1.78
Recovery in supernate	78.1%	77.5%
Total recovery	89.4%	91.0%
Succinate-grown cells		
Supernate	9.70	10.22
3.0-ml. wash	1.47	2.15
Recovery in supernate	77.4%	77.5%
Total recovery	89.1%	93.9%

Standard solution: 15 min., 12.53; 60 min., 13.18.

Equilibration of High Concentration of Succinate; Volume of Wash Varied

In agreement with the results at 3° C. under air, the two types of cells did not differ significantly in succinate taken up at 30° C. under helium (Table III). With the higher concentrations of succinate, the quantity of succinate recovered by washing the centrifuged cells in various volumes of buffer could be compared to determine whether it followed the equilibrium between external solution and cells (either intracellular water or cell substance) indicated by the equilibration with succinate solution. For example, in Table IV 66% of the succinate remained in the supernate from the succinate-grown cells. When washed with three volumes of buffer solution each equal to the volume of succinate solution (4.0 ml.), the percentage of the total compound recovered in succeeding washes should follow a first order reaction; that is, 66% of 34% = 22% in the first wash, 8% in the second wash, etc. This would be true either if equilibration with intracellular water or if a

TABLE III

EQUILIBRATION OF HIGH CONCENTRATION OF SUCCINATE AT 30° C. UNDER AIR OR HELIUM

5.0 gm. of cells; 5.0 ml. 400 μM./ml.	Helium, 15 min.*			Air, 15 min.*		
	Assay	% Recovery		Assay	% Recovery	
		Observed	Calc.†		Observed	Calc.†
Sucrose-grown cells						
Supernate	132.1	67.6	—	133.0	67.7	—
5.0-ml. wash 1	38.8	19.9	21.9	38.1	19.4	21.9
" " " 2	13.4	6.9	7.1	13.7	7.0	7.0
" " " 3	4.2	2.1	2.3	3.8	1.9	2.3
Succinate-grown cells						
Supernate	129.5	66.2	—	113.4	57.7	—
5.0-ml. wash 1	40.6	20.8	22.4	33.3	17.0	19.5
" " " 2	15.4	7.9	7.5	13.9	7.1	6.6
" " " 3	5.2	2.7	2.6	3.8	1.9	2.2

** Assay values are reported in μM./0.5-ml. sample. Standard solution: helium, 195.5 μM.; air, 196.4 μM.*

† Theoretical recovery in washes is calculated from percentage recovery in the supernate, as explained in the text.

TABLE IV

EQUILIBRATION FOR HIGH CONCENTRATION OF SUCCINATE AT 3° C. WITH VOLUME OF WASH VARIED

4.5 gm. of cells; 4.0 ml. 400 μM./ml.	Air, 30 min.*		
	Assay	% Recovery	
		Observed	Calc.
Sucrose-grown cells			
Supernate *A*	135.7	69.1	—
4.0 ml. wash 1	39.0	19.9	21.4
" " " 2	12.6	6.4	6.6
" " " 3	4.2	2.1	2.0
Total wash	55.8	28.4	30.0
Supernate *B*	133.0	67.7	—
12.0-ml. wash	50.4	25.7	26.9
Supernate *A*	130.4	66.4	—
4.0-ml. wash 1	39.6	20.2	22.3
" " " 2	13.8	7.0	7.5
" " " 3	6.3	3.2	2.5
Total wash	59.7	30.4	32.3
Supernate *B*	130.4	66.4	—
12.0-ml. wash	55.4	28.2	28.8

** Assay values are reported in μM./0.5-ml. sample, except that the values for 12.0-ml. washes are multiplied by three for comparisons with 0.5-ml. aliquots of the other (4.0-ml.) samples. Standard solution: 196.4 μM.*

uniform adsorption (which would be proportional to the concentration of external succinate solution) obtained. As seen in Tables III and IV, the experimental recovery in washes showed a reasonable agreement with the calculated values. Theoretical recovery for washes of succinate-grown cells after equilibration at 30° C. under air was calculated as follows: Under helium 66.2% of the succinate appeared in the supernate and 33.8% remained with the cells. After oxidation under air 57.7% was recovered in the supernate; it was assumed that a simple proportion would hold and 29.5% would be present with the cells. Then, 66.2% of 29.5 = 19.5%.

An additional check on equilibration with buffer solutions was made by comparing three 4–ml. washes with one 12–ml. wash. Whether the succinate was diffused into the cell or merely adsorbed onto it, more succinate should be recovered in the three small washes, although the theoretical values for the 12–ml. washes should differ. Assuming diffusion, the effective volume of intracellular water equilibrating with the succinate solution is calculated from the percentage of succinate remaining in the supernate, then the distribution between this volume and 12 ml. is calculated. Assuming uniform adsorption proportional to the concentration of succinate, the amount of succinate remaining on the cells after they are mixed with 4 ml. of buffer should be three times that remaining after they are mixed with 12 ml. Although theoretically the calculations on the two assumptions offer a method for distinguishing between the two mechanisms, with the relative volumes that it is feasible to use in these experiments, the difference between the calculated values is too small. For example, in Table IV the values calculated for succinate-grown cells are 28.8% for diffusion and 29.8% for adsorption.

In the experiment two 4.5-gm. portions each of sucrose-grown and succinate-grown cells were exposed to 4.0 ml. of the succinate solution, then one of each pair was washed with three 4.0–ml. portions of buffer and the other with one 12.0–ml. portion. Again, experimental values agreed reasonably well with calculated. Even though the data do not distinguish between diffusion and adsorption, they do show that, if the latter process is occurring, the succinate is readily removed from the cell substance into the washing medium, and that the adsorption is uniform and proportional to succinate concentration in the external medium. Although radioactive succinate would provide a more sensitive assay, it is questionable whether such a refinement is warranted since the limiting factor in the accuracy is probably not the assay but the washing procedure.

Equilibration With Various Concentrations of Succinate

To determine total recovery, in later experiments, the sucrose-grown and succinate-grown cells were compared by assay without washing. Table V shows a 30-min. equilibration with five concentrations of succinate solution at 3° C. A lower ratio of cells to volume solution (2.5 gm. and 5.0 ml.) was used to facilitate rapid mixing. Assay values are reported in μM./5.0 ml.

supernate or of standard succinate solution and μM./2.5 gm. of cells, instead of μM./0.5-ml. sample as in previous tables, since total recoveries are calculated from the total cell mass rather than from 0.5-ml. assay samples of the buffer washes. Uptake did not differ significantly between the two types of cells at any of the concentrations.

TABLE V

EQUILIBRATION WITH VARIOUS CONCENTRATIONS OF SUCCINATE AT 3°C. UNDER AIR

2.5 gm. of cells; 5.0 ml. solution; 30 min.	Concentration of equilibrating solution in μM./ml.				
	20	40	80	160	400
	Standard solution				
	102.7*	198.2	395.5	796.4	1964
Sucrose-grown cells					
Supernate	83.9	161.6	317.0	632.1	1554
Cells	16.4	34.2	71.4	153.6	381.3
Recovery in supernate	81.7%	81.5%	80.2%	79.4%	79.1%
Total recovery	97.7%	98.8%	98.2%	98.7%	98.5%
Succinate-grown cells					
Supernate	81.3	157.1	312.5	621.4	1580
Cells	16.9	36.5	76.8	149.1	383.0
Recovery in supernate	79.2%	79.3%	79.0%	78.0%	80.4%
Total recovery	95.6%	97.7%	98.4%	96.8%	99.9%

** Assay values are reported in μM./5.0 ml. standard solution or supernate, or μM./2.5 gm. of cells.*

Discussion

A dissolved substance may penetrate into a microbial cell by a process of simple diffusion across a cell membrane, or by an active process requiring an expenditure of energy by the cell, and usually associated with metabolism. (See Osterhout (9) and Rosenberg and Wilbrandt (11) for discussion of the theory of transport systems.) Under conditions where metabolism does not occur, the cell surfaces of adapted and unadapted azotobacter cells do not differ in "affinity" for succinate, so far as can be determined by using masses of cells. Although the data do not distinguish between equilibration with intracellular water and adsorption, they indicate that a change in the surface or membrane of the adapted cells that would allow a more rapid diffusion of succinate across the osmotic barrier has not occurred. The equilibration data agree with results of manometric experiments in which a pre-exposure of the cells to succinate at 3° C. under air or at 30° C. under helium did not reduce the length of time required for adaptation (13). Evidently, any permeability changes during adaptation of *Azotobacter vinelandii* to succinate must be associated with metabolism and its concurrent yield of energy.

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A COMPARISON OF MICROBIAL ACTIVITY IN AN ONTARIO FOREST SOIL UNDER PINE, HEMLOCK, AND MAPLE COVER[1]

By F. E. Chase[2] and G. Baker[3]

Abstract

Soil samples, taken by horizons, were obtained from the University of Toronto Forest near Dorset under stands of maple, hemlock, and pine. Counts of bacteria, actinomycetes, and fungi made by the plate method indicated that in general the organic layer contained the largest population, and also that in the organic layer under conifers the fungi exceeded the combined counts of the other two groups, whereas under maple the bacteria predominated. Using the perfusion method, nitrification did not occur to any extent in these forest soil samples except when lime was added, and even then nitrification started very slowly unless a few crumbs of garden soil were added, presumably as a source of active nitrifying bacteria.

Introduction

The increasing demand for products of our forests has intensified interest in the art of forest management. The realization that regeneration of desirable species is fraught with uncertainty has emphasized the need for a better understanding of those factors which contribute to the success or failure of maintaining selected species in the complex forest environment. One of the environmental factors which has received very little attention, particularly on this continent, is that of the microorganisms in the soil.

Russell recently discussed the characteristics of soils developed under forest conditions, particularly the humus layer (15). He states that mor formation typically occurs under coniferous forest growing on acid soils low in calcium; that in a typical mor, fungi are often considered the most important group (though proof is lacking); and that nitrifying bacteria are typically absent or present in very small numbers, so that mineralized nitrogen tends to appear as ammonia rather than nitrate. In contrast, a mull formation typically develops under deciduous or mixed forests on less acid soils reasonably supplied with calcium. Bacteria are probably the most important microbial agents, and with nitrifying bacteria present inorganic nitrogen is usually found in the form of nitrate rather than ammonia. However, Russell points out that while mor formation typically occurs under coniferous forest, exceptions do occur, and thus mull may be found associated with conifers, and likewise mor sometimes develops under deciduous forest. Thus, while typical associations suggest the general conditions that one may anticipate under coniferous or deciduous forests, since exceptions occur it becomes necessary to investigate conditions in new areas, rather than risk making generalizations.

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Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Canada. Based on a paper presented at the Guelph meeting of the Canadian Society of Microbiologists in June, 1953.
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This paper presents the results of microbial studies made on soil horizons developed from granitic materials of the Pre-Cambrian shield under different species of tree cover, but with other environmental factors being similar.

Experimental Material

Description of Forest Soil

Soil samples were obtained from the University of Toronto Forest (8) within a five-mile radius of the Forest Rangers' School at Lake St. Nora, which is on Ontario Provincial Highway 35, approximately 18 miles south of the western entrance to Algonquin Park. Three locations were selected, having similar site classifications which include such factors as slope, drainage, exposure, ecoclimate (7), but representing different tree cover. Samples were taken by horizon from a pit at each of the sites chosen, and are described below:

Maple Samples

The maple stand was on somewhat excessively drained sandy loam of the brown podsolic soil type (little or no leached layer). The Pre-Cambrian granite bedrock occurred at a depth of 28–30 in.

Maple A_{01}, A_1.—1 to 3 in. of fibrous mor, fairly well decomposed over a trace to $\frac{1}{4}$ in. of a crumblike layer consisting largely of organic matter but with some mineral.

Maple B_2.—10 to 12 in. of silty sand with humus (not iron) colloids.

Maple B_{2g}.—loamy sand, incipiently gleid, between the B_2 and bedrock.

Hemlock Samples

The hemlock stand was on a physiographic site similar to the maple, except the soil was a weak podsol.

Hemlock A_{01}.—$\frac{3}{4}$ to $1\frac{1}{2}$ in. of fairly well decomposed leaf litter of the fibrous mor type.

Hemlock A_1, A_2.—a trace of melanized humus combined with $\frac{1}{2}$ to 2 in. of leached sandy loam, gray in color, with considerable infiltrated humus.

Hemlock B_2.—4 to 6 in. of silty sand, cinnamon brown in color with some iron and humus colloids.

Pine Samples

The pine stand was on an excessively drained sand of podsol characteristics.

Pine A_{01}.—$\frac{1}{4}$ to $\frac{3}{4}$ in. of fibrous mor, partially decomposed and predominantly of pine needle litter.

Pine A_1, A_2.—a trace of sand combined with a trace to 1 in. of leached sand with considerable infiltrated humus.

Pine B_2.—7 to 8 in. of dark brown sand with iron and humus coating (not colloidal).

Chemical Properties of Soil Samples

As soon as the samples were brought to the laboratory, soil pH was determined using a Beckman pH meter and the paste method of Reed and Cummings (13). A portion of each sample was air-dried, and set aside for the following tests: total nitrogen was measured by the micro-Kjeldahl method; organic matter was measured by the wet cumbustion method of Walkley (20), but with ortho-phenanthroline indicator being substituted for the diphenylamine originally used (6); and total exchange capacity was determined by the method of Schollenberger and Simon (16). Results are presented in Table I, in which the feature of greatest interest is the similarity in soil pH under maple cover to that under the conifers. It is also obvious that both total nitrogen and total exchange capacity are closely associated with the quantity of organic matter.

TABLE I

CHEMICAL CHARACTERISTICS OF THE HORIZONS OF A FOREST SOIL UNDER THREE SPECIES OF COVER

Tree cover	Horizon	pH	Organic matter (%)*	Total nitrogen (%)*	Total exchange capacity (m.e. per 100 gm.)*
Maple	A_{01}, A_1	4.28	23.	0.76	34.3
	B_2	4.75	9.6	0.28	17.8
	B_{2g}	5.35	5.2	0.35	13.2
Hemlock	A_{01}	4.20	65.	1.32	93.7
	A_1, A_2	3.92	1.4	0.22	7.6
	B_2	5.31	6.4	0.13	20.5
Pine	A_{01}	4.49	19.	0.44	68.7
	A_1, A_2	4.55	3.7	0.48	7.6
	B_2	5.45	2.8	0.11	8.1

* *Corrected to oven-dry weight.*

Enumeration of Soil Microorganisms

Studies of microbial populations in European forest soils have revealed a tendency for bacteria to predominate under deciduous forest and fungi under coniferous forest; this is shown in several reviews of the subject (4, 11, 15). Similarly, working with forest soils in New York, Cobb (3) found greater numbers of bacteria and actinomycetes in the topsoil of deciduous forest, while the comparable layer beneath hemlock contained the larger population

of fungi. Since the pH values of these topsoils were similar, there was no apparent relationship between acidity and numbers of the various groups of organisms present.

In the present investigation the plate method was used to obtain an estimate of the aerobic population in the different horizons under the three types of tree cover. Unfortunately the plating could not be made until one week after the samples were taken. Suitable dilutions were used to inoculate triplicate plates of soil extract agar (10), the plates being incubated at 28° C. for one week, at which time bacteria and actinomycetes were counted. From the same dilution series, triplicate plates of rose bengal medium (17) were prepared and from these plates fungi were counted after four days at 28° C.

The results are shown in Table II. The most interesting feature was the preponderance of bacteria in the maple organic layer, almost equal to the actinomycete and fungus counts combined; whereas in the coniferous organic layer, fungus counts exceeded the combined bacterial and actinomycete populations. While this favoring of bacteria by deciduous litter, and fungi by coniferous litter, is in accord with the results of workers elsewhere (3, 4, 15), it is apparent from Table I that these results could hardly be due to a pH effect. The selective action appears to be due to some characteristic of the litter layer other than pH, such as the nature of the organic residue, as suggested by the work of Vandecaveye and Katznelson (18). Except for the relatively large actinomycete populations in the second levels of the maple and hemlock samples, the microbial populations were quite uniform throughout the second and third levels.

TABLE II

PLATE COUNTS OF MICROORGANISMS IN THE HORIZONS OF A SOIL UNDER THREE TYPES OF FOREST

(In hundred thousands per gram oven-dry soil)

Tree cover	Horizon	Bacteria	Actinomycetes	Fungi
Maple	A_{01}, A_1	4.2	2.8	2.6
	B_2	1.0	3.5	1.7
	B_{2g}	0.7	1.0	0.1
Hemlock	A_{01}	3.5	4.6	13.1
	A_1, A_2	0.7	3.4	1.0
	B_2	0.7	0.4	1.0
Pine	A_{01}	2.6	7.4	11.8
	A_1, A_2	1.0	0.7	1.5
	B_2	1.4	2.3	0.1

Nitrifying Capacity

The significance of the nitrification process in forest soils is a topic which does not appear to be well defined. Thus Wilde (21) considers it of importance chiefly when dealing with deciduous lime-loving trees that require nitrogen in the form of nitrates, but not for acidophilous conifers which readily utilize nitrogen as ammonia and possibly amino acids. On the other hand, Lutz and Chandler (11) state that little is known about the nitrogen nutrition of forest trees other than that they depend principally on nitrate and ammoniacal forms. They further conclude that from present evidence there is little reason to maintain that nitrate nitrogen is superior to ammonia nitrogen. It seems that such divergent views could arise according to whether emphasis were placed on the typical associations in mor and mull (see Introduction), which would support the pattern suggested by Wilde, or upon the exceptions, which could lead to the different conclusions reached by Lutz and Chandler.

Since the samples in the present study included the typical combination of mor with conifers, and the less common association of mor with maple, a study of the nitrifying capacities was considered of especial interest.

Methods

The perfusion method of Lees and Quastel (9) was selected to determine the nitrifying capacity of these samples. Perfusion units of the type described by Audus (1) were used. The soil samples were air-dried and sieved to separate the 1 to 4 mm. crumb fraction which was used in the units. Portions of each sieved sample, ranging from 10 to 40 gm. depending upon volume weight, were placed in separate units and perfused with 200 ml., (*a*) of distilled water—to ascertain the nitrification of soil nitrogen, and (*b*) of $N/50$ ammonium sulphate—to determine nitrifying capacity in the presence of added ammonium-nitrogen.

Because of the strong acidity of these soil samples, it was not expected that much nitrification could occur without pH adjustment, though a trial was considered necessary since the limiting pH for nitrification has been reported as low as 3.7 (19) and the samples were all above this. In this first test no nitrate appeared in four weeks, hence a second trial was prepared and calcium carbonate added to the soil in the units in amounts which raised the initial pH to range from 6.2 to 7.5. Table III shows the quantities of soil and lime used; and to give a clearer conception of this lime treatment, its equivalent has been expressed as tons of lime per acre per inch of horizon in the forest. It must be emphasized that accurate volume weight measurements of the horizons were not made, so that the forest requirements are presented as approximations only. The liming treatment was only partially successful, because in 7 out of 36 units the pH of the perfusate dropped to 5.0 or below by the end of the 28-day experimental period. This degree of acidity only reoccurred in units containing added ammonium sulphate. To determine whether the introduction of active nitrifying bacteria would hasten nitrification in these soil samples, paired units of each sample were prepared, and one

TABLE III

LIME TREATMENT OF FOREST SOIL SAMPLES IN PERFUSION UNITS, AND ESTIMATED FOREST EQUIVALENT

Tree cover	Horizon	Amount in each perfusion unit		pH 2 days after lime	Forest equivalent of lime treatment† (tons per acre-inch)
		Soil*, in gm.	Calcium carbonate, in gm.		
Maple	A_{01}, A_1	20	0.60	6.4	2.5
	B_2	40	0.80	7.5	3.0
	B_{2g}	40	0.16	7.0	0.6
Hemlock	A_{01}	10	0.40	6.2	1.7
	A_1, A_2	40	0.20	6.7	0.8
	B_2	40	0.16	6.2	0.7
Pine	A_{01}	10	0.40	6.2	1.7
	A_1, A_2	25	1.00	6.2	4.0
	B_2	40	0.20	6.9	0.8

** Amounts represent approximately equal volumes, hence indicate ratio of volume weight of samples.*

† Calculation based on relative volume weights of different profile samples; lowest levels assumed roughly equivalent to agricultural field soil (1.3 gm. per cc.).

TABLE IV

CHANGES IN pH OF PERFUSATE DURING NITRIFICATION IN LIMED FOREST SOIL WITH AND WITHOUT ADDED NITROGEN

Tree cover	Horizon	Perfused with			
		Water		*N*/50 Ammonium sulphate	
		14 days	28 days	14 days	28 days
Maple	A_{01}, A_1	6.3	5.3	6.6	5.6
	B_2	7.4	7.4	7.5	6.2
	B_{2g}	7.2	6.4	5.4	5.2
Hemlock	A_{01}	6.8	5.8	6.6	6.2
	A_1, A_2	5.6	5.2	6.6	4.6
	B_2	6.0	5.7	5.0	4.6
Pine	A_{01}	6.5	6.3	6.6	5.0
	A_1, A_2	7.4	7.8	6.9	6.8
	B_2	6.6	6.0	5.3	4.8

unit of each pair was inoculated by placing approximately 0.1 gm. of fresh garden soil on the surface of the forest soil in the sample tube.

Progress of nitrification was followed at three- to four-day intervals by estimating nitrate-nitrogen using the method of perfusate analysis described by Chase (2), but with the amount of hydrogen peroxide used to oxidize the

organic matter increased (to 0.5 ml. of 10% hydrogen peroxide) for the samples containing high concentrations of organic matter. Nitrite-nitrogen in the perfusate was determined using the method of Rider and Mellon (14). Seldom did more than a trace of nitrite appear in the distilled water perfusate; and though nitrite often accumulated for a time in the ammonium sulphate perfusate, this did not alter the general trends found in the nitrate data, and hence only the latter are reported in this paper. The pH of the perfusate was measured with a Beckman pH meter each time other analyses were made.

Results

The lime treatment and pH of the perfusates at the middle and end of the 28-day period are presented in Table IV (the pH two days after liming was included in Table III). The results indicate the heavy lime applications necessary to adjust the pH of these acid soils close to neutrality, and maintain

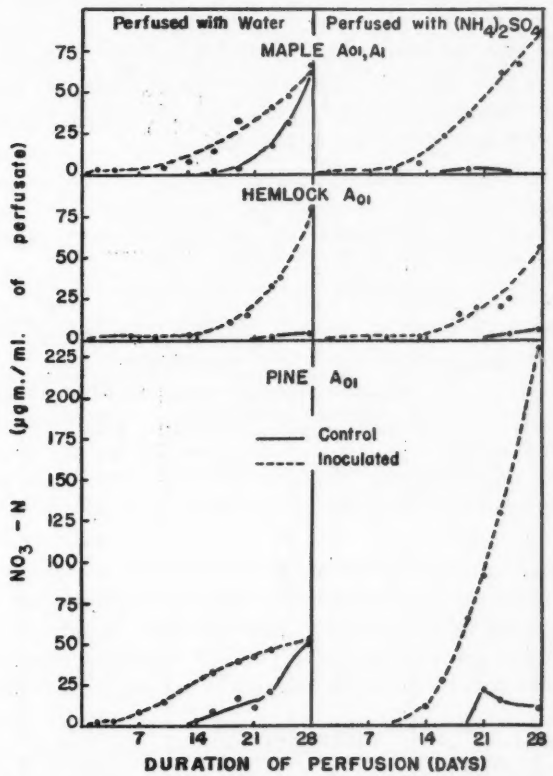

FIG. 1. Effect of inoculation with garden soil, and of adding ammonium-nitrogen, on nitrification in the organic layer of limed forest soil developed under three types of tree cover. No nitrification occurred when lime treatment was omitted.

them against the acid produced, when only the native soil nitrogen was nitrified; even more lime should have been used in those units perfused with ammonium sulphate solution, because of increased acid production.

The effects of inoculation and of adding ammonium-nitrogen on nitrification in the limed samples of the organic layers are illustrated in Fig. 1. The graphs show that inoculation started the nitrification process a week or two earlier than occurred in the uninoculated units. This advantage was maintained in the units containing added ammonium-nitrogen throughout the 28-day experimental period, but not in the units perfused with water. In two out of three of the latter units the initial advantage of inoculation had disappeared by the 28th day, suggesting that at this time ammonification of soil nitrogen had become the rate-limiting factor, rather than an inactive or small population of nitrifying bacteria. No reason can be given for the apparent inhibition of nitrification that occurred when the uninoculated organic layer was perfused with ammonium sulphate solution rather than water. This unexpected inhibition did not appear in any of the mineral samples of the three profiles. This is evident in Table V, which shows the nitrate-nitrogen produced in all the layers after 28 days. In general these results indicate that in the mineral layers, both inoculation and the addition of ammonium-nitrogen stimulated nitrification.

TABLE V

EFFECT OF INOCULATION WITH GARDEN SOIL ON NITRIFICATION IN LIMED SAMPLES OF FOREST SOIL WITH AND WITHOUT ADDED NITROGEN

Tree cover	Horizon	Nitrate-nitrogen formed in 28 days (μgm. per gm. air-dry soil)			
		Water perfusate		Ammonium sulphate perfusate	
		Control	Inoculated	Control	Inoculated
Maple	A_{01}, A_1	670	630	0	880
	B_2	150	135	180	1220
	B_{2g}	6	0	400	235
Hemlock	A_{01}	60	1620	120	1160
	A_1, A_2	85	135	90	255
	B_2	12	65	50	325
Pine	A_{01}	1080	1080	220	4660
	A_1, A_2	284	208	456	2070
	B_2	25	15	115	220

Discussion

This investigation of the way in which type of tree cover may influence the microorganisms in forest soil, was undertaken primarily as a survey, to discover whether a more intensive program would be justified, and if so, the most promising lines to follow. Nothing unusual was observed in the limited chemical studies made, and the association of a larger bacterial population in the organic layer under maple, and fungus populations under conifers, was similar to that found by others, both on this continent (3) and elsewhere (4). The occurrence of a predominant bacterial population beneath maple, in spite of the formation under maple of an acid mor commonly associated with conifers, certainly suggests that the nature of organic matter must be more important than pH. In this connection our findings in Ontario soil are in accord with results of other workers using soils from Idaho (18), and New York (3).

The nitrification studies showed that in the profile samples of this Ontario forest soil, lime applications were necessary before any nitrification occurred. Some improvement in nitrate production during laboratory studies involving lime treatment of forest soils has been reported by Powers and Bollen (12). The stimulation obtained from inoculation suggests that very few nitrifiers can be present normally. Furthermore, the amount of nitrate-nitrogen produced from native soil nitrogen, in the organic layers especially, indicates that appreciable quantities of nitrogen should be readily available, at least if the pH were adjusted by lime treatment. Also the increased rate of nitrate production that occurred when ammonium-nitrogen was added showed that the horizons in these profiles were capable, under ideal conditions, of a rate of nitrification that appeared to be well in excess of the ability of the organisms in the limed soil to release ammonia from the soil organic matter.

Since nitrification did not occur unless the samples were treated with lime, it seems that only organic and ammonium-nitrogen would normally be found in these profiles. It has also been observed that maples tend to be unthrifty in this region, but whether this is due to lack of nitrate-nitrogen, to the strong acid reaction of the soil, or to some other nutrient deficiency or environmental factor is not known.

While no attempt has been made to apply the findings to problems of forest regeneration, the subject of nitrogen transformations and nutrition of trees does appear worthy of further study. Actually, this problem is already receiving attention elsewhere, Gessel (5) having reported improved growth of Douglas fir following the use of nitrogen fertilizer in natural stands.

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EXPERIMENTS ON THE METHANE BACTERIA IN SLUDGE[1]

By R. L. Mylroie[2] and R. E. Hungate

Abstract

An agar culture method for determining the numbers of methanogenic bacteria in sludge was developed. Hydrogen and carbon dioxide were provided as substrate and palladium as a reduction catalyst. Sodium sulphide could also be used for reducing the medium. A methanogenic organism could be identified by correlating methane production with presence of a particular type of colony which was the most abundant. Fifteen strains which were pure cultured were all similar and were classified as *Methanobacterium formicicum*. The culture count for methane bacteria in sludge ranged between 10^5 and 10^8 per ml. The rate of fermentation and the efficiency of conversion of substrate into cell material were studied. Presence of an acetate-fermenting methanogenic organism was established but no pure cultures could be obtained. *Methanobacterium formicicum* appeared to be the only methane producer growing in the dilution series used for counts.

The occasional malfunctioning of sewage fermentations (8) emphasizes the need for an understanding not only of the abnormal but also the normal microbial and biochemical processes involved. Since methane is an important product of the sludge fermentation, the factors concerned with its production warrant study. The numbers of methane bacteria in sludge capable of attacking various substrates were estimated by Heukelekian and Heinemann (5, 6), using liquid dilution cultures, but the organisms concerned were not isolated. Studies on the methane bacteria have usually employed enrichment as a preliminary procedure for obtaining active cultures (4, 12, 14), and in those cases where pure cultures were isolated they also were derived from enrichments (2, 11, 13).

Since during the enrichment process the kinds and numbers of methane bacteria may undergo significant changes, in the present study an attempt was made to isolate the methane bacteria directly from the "natural" source, to develop a technique for counts in agar dilution cultures, and to ascertain the numbers in which the methane bacteria occurred.

Preliminary Experiments

As a first approach it seemed logical to use as substrate the medium in which the organisms were growing. Active sludge was serially diluted into a medium composed of 60 ml. of sludge liquor plus 40 ml. of inorganic salts of the following percentage composition (1): NH_4Cl 0.1, K_2HPO_4 0.04, KH_2PO_4 0.04, $MgCl_2 \cdot 6H_2O$ 0.01, and Difco agar 1.5. The sludge liquor was obtained from the secondary digestion tank of the Pullman municipal plant, neutralized with dilute sulphuric acid, and centrifuged. The supernatant liquid, which

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was light brown in color, was added to the inorganic salts. Oxygen was displaced with carbon dioxide, which, with added sterile sodium carbonate, provided buffering action. Sodium sulphide was added as a reducing agent.

Anaerobic techniques similar to those of Hungate (7) were employed and the serially diluted tubes were incubated at 38° C. After two months the gas in the culture tubes was transferred by removing the stopper from the inverted tube under water and pouring the collected gas into a second tube filled with water and furnished with a stopcock at the upper end for transferring the gas to a semimicro modification of the Newcomer-Haldane gas analysis apparatus.

Analyses of these initial cultures failed to disclose any indications of methane. Substitution of cysteine for sodium sulphide did not improve the results. Addition of rumen fluid, yeast extract, acetate, or propionate did not appreciably increase the methane production, though traces of methane could occasionally be detected in the tubes of lower dilution. Use of sterilized fresh sludge as a medium gave no better growth. Modifications of the temperature and the salt concentration, using fresh sludge from the sewage plant, failed to increase the rate of methane production.

The ability of some of the methane bacteria to use hydrogen and carbon dioxide (3, 11, 12) suggested the use of these gases as an energy source, so parallel series containing 95% hydrogen – 5% carbon dioxide and 95% nitrogen – 5% carbon dioxide, respectively, were set up. Cysteine was the reducing agent. After inoculation and incubation the amount of methane in the hydrogen tubes significantly exceeded that in the nitrogen tubes, the first encouraging results which had been obtained. The composition of the culture gas was changed to 80% hydrogen – 20% carbon dioxide with greater resulting quantities of methane.

With this gas mixture, using either the inorganic salts alone or with added 60% sludge liquor, serial dilutions of sludge showed occasional tubes with methane. Addition of activated charcoal, starch, or citrate did not improve the dependability of growth, suggesting that absence of growth was not due to adsorbable toxic materials.

A correlation was noted between the presence of methane and colonies so profusely branched that they became villous in appearance. Subcultures from these colonies gave sporadic growth, with cysteine as a reducing agent. When growth did occur the tube was found to contain methane, indicating that the organism forming the arborescent colony was a methane producer.

The oxidation–reduction potential of the medium remained as an important factor possibly concerned with the irregular growth obtained. The use of hydrogen in the culture medium suggested that addition of a catalyst might give the low potential of the hydrogen electrode. Small amounts of palladium black were included in the medium and other reducing agents were omitted. This procedure initially gave consistently good growth and a pure culture of an organism identified as *Methanobacterium formicicum* (11) was obtained. Less satisfactory growth was later obtained and after much experimentation

it was found that addition of palladium chloride (either as a finely divided suspension or dissolved in hydrochloric acid) just before the sterile medium in the tubes was melted prior to inoculation, gave consistent growth. The palladium chloride was reduced by the hydrogen during the melting of the agar.

After the pure culture was obtained, a careful study of the suitability of sodium sulphide as a reducing agent showed that in concentrations of $Na_2S \cdot 9H_2O$ between 0.01 and 0.015% growth could consistently be obtained, but not using concentrations of 0.005 or 0.02%. In the initial experiments the optimum range of sodium sulphide concentration had been exceeded.

Final Experiments

As a result of all the preliminary experiments the culture procedure as indicated below was finally adopted. The salt solution containing 0.1% NH_4Cl, 0.04% KH_2PO_4, 0.04% K_2HPO_4, and 0.01% $MgCl_2 \cdot 6H_2O$ in distilled water was sterilized with 1.5% Difco agar in a 500-ml. round-bottom stoppered flask filled with oxygen-free carbon dioxide. After autoclaving, the medium was cooled to 45° C., the stopper removed, and oxygen-free carbon dioxide immediately bubbled into the flask through a sterile Pasteur pipette. Two milliliters of sterile 10% sodium carbonate per 100 ml. of medium were added. The medium was dispensed aseptically in 4.5-ml. aliquots into sterile test tubes using anaerobic procedures. When the medium

TABLE I

CULTURE COUNTS OF *Methanobacterium formicicum* IN SLUDGE

Sample	Date	Source	Reducing agent	Culture count/10^5
1	9–17–51	Primary tank Pullman	Cysteine	250
2	10–27–51	" " "	"	5
3	1– 4–52	" " "	Palladium	28
4	4–18–52	" " Moscow	"	3
5	6–11–52	Secondary " Pullman	"	21
6	6–15–52	Primary " "	"	19
7*a*	6–25–52	" " "	"	4
7*b*				9
8	7–15–52	" " "	"	1
9*a*	8– 8–52	" " "	"	16
9*b*				1
10	9– 4–52	" " "	"	290
11	9–17–52	" " "	"	63
12	9–25–52	" " "	"	28
13*a*	10– 4–52	" " "	"	42
13*b*			Sodium sulphide	970
14*a*	10–21–52	" " "	"	730
14*b*			"	540
14*c*			Palladium	880
14*d*			"	930
15	10–23–52	" " "	"	130
16	10–25–52	" " "	"	680
17	6–14–52	" " Dayton	"	8
18	7– 7–62	" " "	"	16

had solidified, the palladium chloride (0.1 ml. of a 0.5% suspension) was added to each tube and the carbon dioxide immediately replaced with hydrogen. The medium was remelted in a boiling water bath, again cooled to 45° C. and then inoculated. The inoculum was serially diluted by transferring 0.1 ml. each time. During these operations oxygen-free carbon dioxide rather than hydrogen was passed through the tubes in order to reduce the danger of an explosion. After inoculation the carbon dioxide was replaced with a mixture of 80% hydrogen – 20% carbon dioxide. The tubes were solidified as roll tubes and incubated at 38° C.

Counts of the organisms in sludge were made at various times during a period of one year with results as shown in Table I.

The counted colonies showed the appearance typical of *M. formicicum*. They were more abundant than any other type present. The counts in samples 7, 9, 13, and 14 give an idea of the reproducibility of the culture counts. It is evident that a considerable error was still involved. A number of the counts obtained are significantly higher than those previously reported for sludge and in sample 14, which showed high counts, the agreement between duplicate series is fairly good.

Characteristics of the Isolated Organism

Fifteen strains were obtained in pure culture but since they were similar in both cell and colony morphology, the detailed characteristics of only one strain were studied.

Morphologically, the cells were Gram-negative rods 0.4 μ in diameter and variable in length, often joined into chains (Fig. 1). No motility or spore production was observed. Colonies in deep agar were composed of fine, profusely branched filaments which gave young colonies an arborescent appearance. Older ones appeared villous.

The optimum temperature range was between 38° and 45° C. with no growth at 55° C. Lower temperatures were not tested.

The effect of pH on growth was studied both at 45° C. and at 38° C. Various acidities were obtained with varying amounts of sodium bicarbonate. The initial pH was determined in an uninoculated control tube containing the same amount of sodium carbonate as the corresponding experimental tube. At the end of the experiment, as a check, on the assumed initial pH, the agar was melted, 80% hydrogen – 20% carbon dioxide was again equilibrated with the liquid, and the pH was measured with a potentiometer. No significant difference from the initial pH was found.

The experiment was set up in triplicate and the tubes examined periodically to determine the time at which colonies could be detected. In the experiment at 38° C. a wider range of pH values was obtained by using 100% hydrogen for pH's above 7.8 and adding sulphuric acid for pH's below 6.0. The results are shown in Table II.

The results with the cultures at 45° C. do not indicate much effect of pH over the range 6.6 to 8.0. It is doubtful whether the limits of pH within

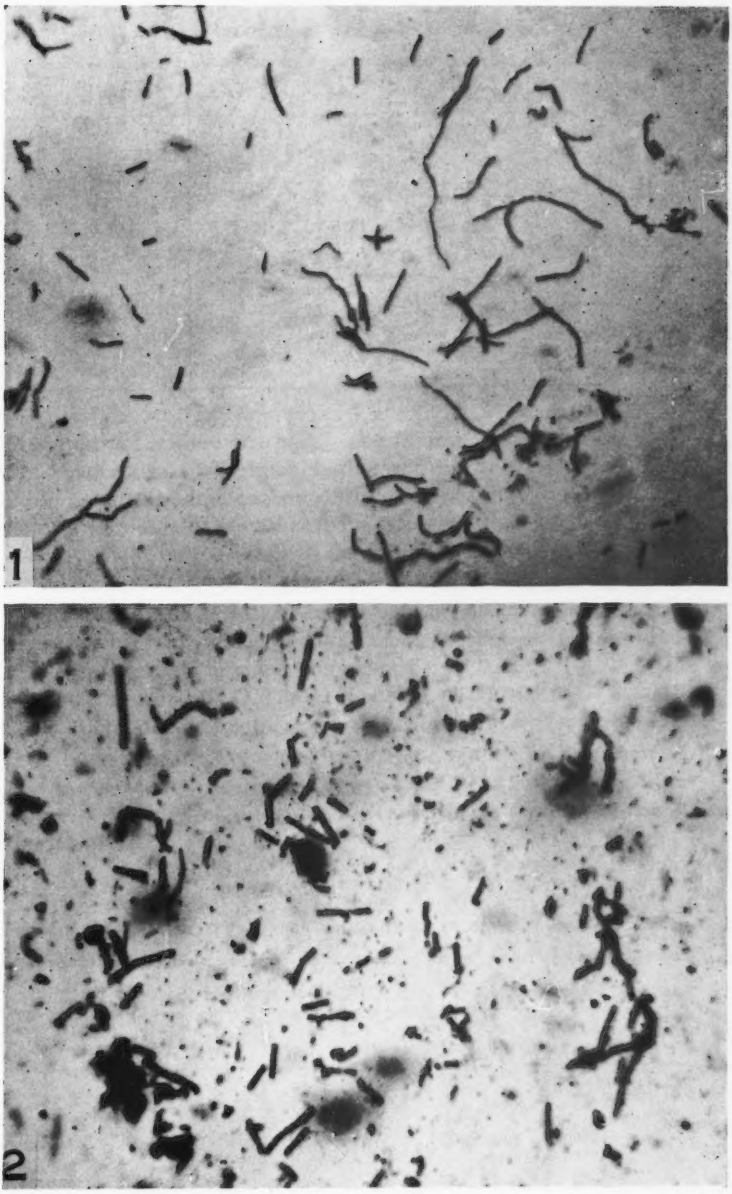

FIG. 1. Photomicrograph of *Methanobacterium formicicum*. Gentian violet. FIG. 2. Photomicrograph of sludge enriched with acetate. Same scale as Fig. 1.

TABLE II

TIME OF APPEARANCE OF COLONIES IN MEDIA AT VARIOUS ACIDITIES

Initial pH	38° C., in days	45° C., in days
5.6	No growth	
6.0	" "	
6.6	7, 5, 7	4, 7, 7
6.8	8, 10, 10	4, 4, 7
7.0	9, 9, 9	5, 5, 8
7.2	8, 8, 9	4, 8, 8
7.4	8, 9, 9	7, 8, 8
7.6	8, 9, 9	4, 8, 8
7.8	5, 8, 8	5, 8, 8
8.0	No growth	5, 7, 8
8.2	" "	
8.5	" "	

which growth would occur were reached. The failure of growth above pH 7.8 in the 38° C. experiments was probably due to the lack of carbon dioxide. At that pH, the amount of free carbon dioxide in solution would be small and if the bacteria were unable to use carbonate or bicarbonate, the amount of growth possibly would be too small to detect. On the acid side, pH 6.6 would appear to be near the limit for growth.

Addition to the pure culture of 0.2% yeast extract, or 25% sludge liquor did not decrease the time for appearance of colonies. The effect of adding the following percentages of inorganic compounds was tested: $CaCl_2$ 0.01, $MnSO_4 \cdot H_2O$ 0.002, $FeSO_4 \cdot 7H_2O$ 0.002, Na_2MoO_4 0.0001, $CuSO_4$ 0.0001, and $CoCl_2$ 0.001. The details for these experiments are given in Table III.

At 38° C. the trace elements appeared slightly to inhibit growth, and at 45° C. they did not support more rapid growth than did the basal medium plus sludge liquor. Addition of sludge liquor caused slight improvement in growth in experiment 2.

TABLE III

TIME REQUIRED FOR FIRST APPEARANCE OF COLONIES IN VARIOUS MEDIA

	Experiment 1		Experiment 2	
	38° C., in days	45° C., in days	38° C., in hours	45° C., in hours
Basal medium	4, 4	4, 4	74, 74	74, 74
Basal + yeast extract	No growth	No growth	No growth	No growth
Basal + sludge liquor	4, 4	4, 5	66, 74	66, 66
Basal + trace elements	4, 4	4, 4	104, 104	66, 74

In other experiments using liquid cultures and hydrogen – carbon dioxide as substrate, sludge liquor, phytone, or added asbestos failed to increase growth significantly. In one experiment growth was obtained in a yeast extract culture but it was no more rapid than in the control.

Tests for utilization of various substrates were run in triplicate. The routine culture method was used except that the percentage of hydrogen was reduced to 10%, the remaining gas being carbon dioxide. This amount of hydrogen provided a suitably low redox potential and supported a small but detectable amount of growth. This medium without any organic additions served as one control and the usual tube with 80% hydrogen as another. In some experiments the cultures were analyzed for methane; in others only growth was observed. The results of adding various organic materials are shown in Table IV.

TABLE IV

UTILIZATION OF VARIOUS SUBSTRATES BY *Methanobacterium formicicum*

Gas phase	Substrates added	Experiment 1 ml. of methane formed			Experiment 2 + indicates growth − indicates no growth		
		a	*b*	*c*	*a*	*b*	*c*
80% H_2 – 20% CO_2	None	2.4	1.2	2.3	+	+	+
10% H_2 – 90% CO_2	None	0.3	0.15	0.3			
"	Ethanol	0.1	0.1	0.25			
"	Methanol				−	−	−
"	Formate	1.9	2.2	3.1	+	+	+
"	Acetate	0.2	0.3	0.0	−	−	−
"	Propionate	0.2	0.3	—	−	−	−
"	Butyrate	0.1	0.2	0.2	−	−	−
"	Lactate	0.2	0.2	—			
"	Glucose	0.15	0.15	0.2			

Steam distillation of the formate cultures showed that the formate had disappeared. In order to test more fully the ability to use acetate or butyrate, the organism was inoculated into the usual 80% hydrogen medium to which acetate or butyrate had been added and the tubes were incubated for one month. The usual abundant growth resulted. Analysis of the tubes for volatile acids disclosed the same amounts as in uninoculated controls, which shows conclusively that neither acetate nor butyrate was utilized by this organism. No substrates other than formate, or hydrogen and carbon dioxide, were found to support growth. Carbon monoxide was not tested. The cell morphology and the substrates fermented are the basis for assigning the organism to *Methanobacterium formicicum* (11).

A Quantitative Culture Experiment

In order to obtain an idea of the maximum rate at which substrate might be converted, a culture in which the gases could be replenished was set up as in Fig. 3. Twelve milliliters of inorganic culture solution inoculated with *M. formicicum* were added to a 70-ml. culture tube containing 80% hydrogen – 20% carbon dioxide. After several days of incubation at 38° C. the gas pressure in the vessel diminished as indicated by the rise of the mercury in arm *A* of the manometer. The changes in level of the mercury were recorded and when necessary the pressure in the flask was restored by first flushing out the system to the 3-way stopcock *C* and then replacing the accumulated methane by flushing hydrogen – carbon dioxide through the culture tube and past the mercury in arm *B* of the manometer. This was done quickly and after a few minutes of equilibration a new reading was recorded.

In this way the pressure changes due to the conversion of hydrogen and carbon dioxide into methane were continuously measured and recorded over a period of 17 days. The initial readings involved a good deal of error but the later larger pressure changes were more accurate. The readings are plotted in Fig. 4. By 297 hours an approximately constant rate of pressure decrease had been reached. It was assumed that the rate at which hydrogen diffused into the culture limited the fermentation rate. At 357 hours the culture was shaken and as can be seen in the figure the rate of fermentation increased markedly and again came to a constant value, the second constant rate being about 10 times as fast as the previous one.

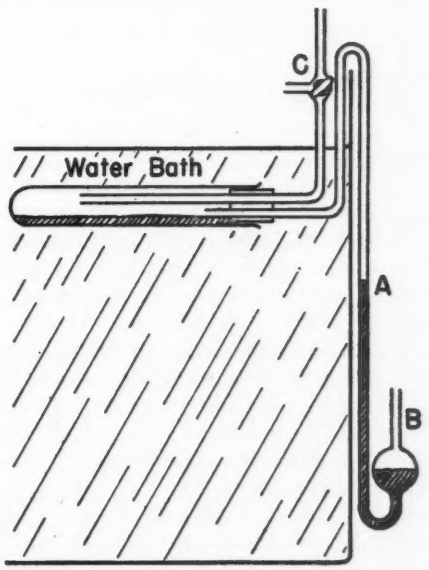

FIG. 3. Apparatus for quantitative study of hydrogen and carbon dioxide utilization.

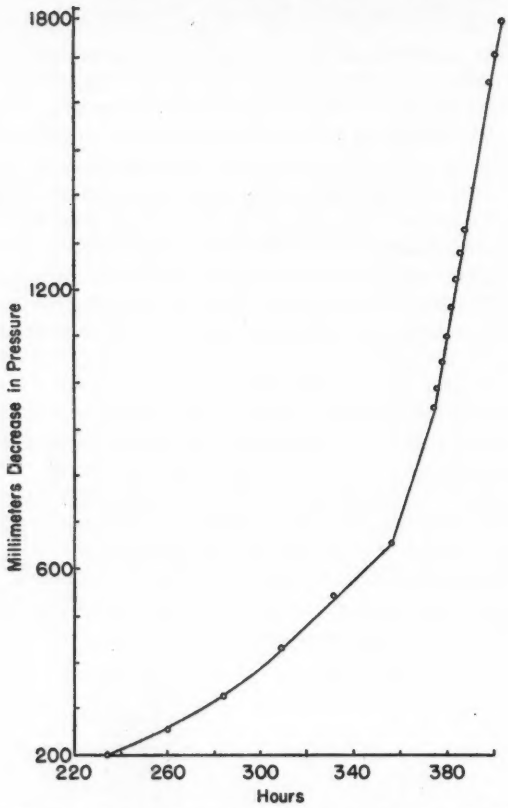

FIG. 4. Pressure changes due to hydrogen and carbon dioxide utilization.

After this maximum rate of fermentation had been reached readings were made at less frequent intervals but the addition of gas was continued for a total period of 563 hours in order to obtain sufficient growth to permit gravimetric determination of the amount of cell material synthesized. The total pressure change was 5530 mm. Hg.

In the 70-ml. vessel containing 12 ml. of liquid culture medium the ratio of the amount of carbon dioxide in the gaseous phase to that in the liquid phase was $\frac{CO_2 \text{ gas}}{CO_2 \text{ liq.}} = 58 \times \left(\frac{p}{760} \times \frac{273}{311}\right) \Big/ \left(12 \times \frac{p \times 0.55}{760}\right)$ where p is the partial pressure of carbon dioxide and 0.55 is the solubility coefficient of carbon dioxide in the culture medium. The fraction of the total gas in the vapor phase would be (7.714/8.714) = 0.885. If the reaction $CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O$ = 68,000 cal. represented the fermentation in the culture, the pressure decrease in the manometer due to carbon dioxide utilization would equal 0.885 of the pressure increase due to methane production, disregarding

the slight solubility of methane in the culture solution. The pressure decrease due to hydrogen consumption would be four times the increase due to methane production. For each unit pressure increase due to methane production, there would be a total decrease of 4.885 due to hydrogen and carbon dioxide utilization, giving a net change of − 3,885 units of pressure. Thus the recorded pressure change on the manometer, in order to give the pressure change due to hydrogen consumption, must be corrected by the factor 4/3.885. The amount of hydrogen used then can be calculated as

$$\frac{4}{3.885} \times \frac{5530}{760} \times \frac{273}{311} \times 58 \text{ ml.} = 381 \text{ ml. or } 17 \text{ mM. of hydrogen.}$$

This indicates utilization of 4.25 mM. of carbon dioxide and an energy liberation of 289 calories. The dry weight of the cell material in the culture at the end of the experiment was 7.1 mgm. Since the weight of the cells in the inoculum was negligible, the energy stored in cell material, if estimated as equivalent to protein, would be 32 calories, or 11% of the energy released in the fermentation. The carbon assimilated, assuming that it composed 50% of the bacterial cells, would be approximately 0.3 mgm. atoms or 7% of the carbon metabolized.

The cells at the end of the experiment may have contained some insoluble sulphates and carbonates but there was no visible indication that the latter constituted a significant fraction of the residue. Any insoluble salts in the residue of bacteria would give an apparent efficiency higher than the actual one. The efficiency of the energy conversion found is of the same order of magnitude as those reported for various aerobic autotrophs (15) and is of interest because it refers to an exclusively anaerobic organism.

Fermentation of Acetate

The demonstration of acetic acid as a product of cellulose fermentation by bacteria in sludge (9), the common occurrence of acetic acid as an end product of many other bacterial fermentations, and the conversion of hydrogen and carbon dioxide to acetic acid in sludge (10) made it probable that this compound also was metabolized in sludge to methane. Failure of *M. formicicum* to attack acetate suggested the presence of other methanogenic bacteria. This possibility was examined by adding acetate to sludge to elicit a greater development of methane bacteria attacking this substrate.

In such cultures an organism distinct in morphology from *M. formicicum* was abundant. It is shown in Fig. 2.

Attempts to obtain pure cultures of this presumed acetivorous methanogenic organism were entirely unsuccessful. Even in initial dilution series from the sludge enrichment, though the lowest dilution occasionally showed methane production from acetate, there was no methane produced in slightly higher dilutions in which the suspected organism was certainly present. On the basis of reports listing *Methanobacterium söhngenii* as capable of attacking acetate it was believed that this was the species active in the acetate enrichments but showing little growth in dilution series.

Discussion

The time available was insufficient to permit testing for methane production all types of colonies appearing in the dilution series in which counts of methane bacteria were made, but two lines of evidence suggested that no methane organisms other than *M. formicicum* were growing and using hydrogen and carbon dioxide in tubes of higher dilution. (1) Tubes positive for methane always showed the typical diffuse colonies of *M. formicicum;* no instances of methane production occurred in its absence. (2) A further check was made by inoculating two parallel dilution series with sludge. The gas used for one series was 80% nitrogen – 20% carbon dioxide and for the other it was 80% hydrogen – 20% carbon dioxide. The only type of colony appearing in the hydrogen tubes and not present in the nitrogen tubes was *M. formicicum.*

The occurrence of *M. formicicum* in large numbers in sludge indicates that it must be of importance in sewage fermentations. The rapid conversion of formate to methane and carbon dioxide when added to sludge (10) is in accord with this view. Other methanogenic bacteria, converting other substrates into methane and carbon dioxide, must also be present.

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STUDIES ON THE FLAGELLA OF ALGAE

V. SEROLOGY OF PARALYZED MUTANTS OF *CHLAMYDOMONAS*[1]

By Rita H. Mintz and Ralph A. Lewin

Abstract

No significant serological differences could be detected between the flagella of wild-type cells of *Chlamydomonas moewusii* and those of paralyzed mutants.

Introduction

Wild-type cells of *Chlamydomonas moewusii* Gerloff bear two flagella which render the organisms motile. After irradiation of wild-type cells with ultraviolet light, mutants have been isolated in which the flagella are apparently paralyzed (4). It was of interest to determine whether any serological differences could be detected between the flagella of wild-type cells and those of paralyzed mutants. In order to eliminate complications due to somatic antigens, experiments were carried out using only cell-free suspensions of flagella.

Material and Methods

(*a*) *Preparation of Antigen*

Cells of both wild-type and of various mutant strains derived from the *minus* mating-type were inoculated into 1 liter of mineral medium (3) in a 2 liter Erlenmeyer flask. Cultures were grown with constant aeration at 23° C. under a light intensity of 450 ft-c. for 13 days. They were then incubated in darkness overnight, in order to reduce the numbers of cells in nonflagellate stages undergoing asexual reproduction. The cells from each culture were concentrated by centrifugation and the bulk of the supernatant fluid removed. The remaining dense suspension, containing *ca.* 10^{10} cells in 10 ml., was adjusted to pH 3.0 with dilute hydrochloric acid, and allowed to stand for five minutes. At this pH the cells shed their flagella, though they remain viable. The mixture was then readjusted to pH 8.0 with sodium hydroxide, and the cell bodies removed by centrifugation for two minutes at 1000 *g*, leaving the flagella in suspension. Sodium chloride was added to a final concentration of 0.85%. The colorless supernatant, containing *ca.* 0.1 mgm. organic nitrogen per ml., was stored at 0° C. All stages in the preparation of this inoculum were performed under sterile conditions.

(*b*) *Preparation of Antibody*

Injections of each antigen were administered in the marginal ear vein of one rabbit in weekly doses increasing from 0.25 to 1.50 ml. for nine weeks.

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Blood was then drawn from the heart, allowed to clot, and centrifuged. The serum was heated in a water-bath at 62° C. for 30 min., preserved with five drops of 5% phenol, and stored at 0° C. until used.

(*c*) *Complement Fixation Test*

Sheep erythrocytes (2% in saline) were employed. Antisheep hemolysin and dehydrated complement were obtained from the Difco laboratories. Suitable dilutions were prepared with 0.85% sodium chloride. Preliminary titrations of hemolysin, complement (2), and antigens (8) were made. The final complement fixation test was then performed (2).

Results

Antigen–antibody interactions were cross-tested, and the results of two experiments are summarized in Tables I and II. The numbers indicate the highest antibody dilutions in each case at which partial or complete hemolysis was observed after incubation at 37° C. for 45 min. Though the antisera of the four rabbits differed in titer, no significant serological differences were observed between the reactions of the various flagellar antigens. The slight difference between the antigen titer of wild-type and paralyzed mutants in Table II was not considered significant.

TABLE I

TITERS OF ANTISERA

Antibody (rabbit serum)	Antigen (flagella suspension)			
	Wild +	Wild −	M.1017 −	M.1041 −
Wild +	16	16	16	16
Wild −	64	64	64	64
M.1017 −	8	16	16	8
M.1041 −	8	8	16	16

TABLE II

TITERS OF ANTISERA

Antibody (rabbit serum)	Antigen (flagella suspension)				
	Wild −	M.336 −	M.1034 −	M.1038 −	M.1043 −
Wild −	16	16	16	16	32

From these results no distinctions could be made between the two mating-types, or between paralyzed mutants and normal swimming cells.

Discussion

For many years serological methods have been extensively employed for the characterization of bacterial strains, and recently paralyzed and fully motile strains of *Salmonella* have been compared by this means (7). In addition, differences as to race and strain in *Paramecium* have been shown to be correlated with serological specificity (9). There have been comparatively few studies of this sort in algae, however. Rosenblat-Lichtenstein (6) reported serological differences between a green strain of *Chlorella protothecoides* Krüger and a spontaneous mutant with reduced pigmentation. Hutner and Provasoli (1) and E. Behrman (unpublished) were unable to demonstrate any serological difference between the two mating-types of *Chlamydomonas moewusii* and this result has been confirmed in the present study.

Lewin (5) has shown that in certain mutants of *C. moewusii* (e.g. M.1017) paralysis may be attributed to impaired synthesis of unidentified factors which, since they are presumably intracellular, are unlikely to give rise to serological differences in the flagella. However, in other mutants (e.g. M.336, M.1034, M.1038, M.1041, and M.1043) no transmissible agent appeared to be implicated. It was among these that serological differences might be expected, indicating changed biochemical characteristics of the flagella themselves. No evidence has yet emerged that differences of this sort exist.

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THE MIGRATING NUCLEUS[1]

By E. S. Dowding[2] and A. Bakerspigel

Abstract

The nuclei of *Gelasinospora tetrasperma* travel out of a mycelium of one sex and into one of opposite sex as rapidly as 10.5 mm. per hour. This occurs not only in pairings producing abundant perithecia but also in pairings that produce few or none. The nuclei migrate from cell to cell in a protoplasmic strand running through the center of the hypha. Nuclei have been observed travelling through their own mycelium. They have been seen passing from one cell, through the pore in the transverse septum, into the next cell. The shape and size of the nucleus fluctuates. Spherical nuclei may change to narrow elongate sinuous bodies, and then resume their spherical shape. Nuclei may migrate in an expanded, contracted, or elongate form.

Introduction

It is well known that many species of fungi possess plus and minus strains. A plus strain or a minus strain growing alone is sterile, but when two such strains grow side by side until they meet they produce fruit bodies along the line of contact. Plus hyphae fuse with minus hyphae; nuclei from the two strains come together to form plus–minus pairs; the members of the pairs conjugate, and so the fruit bodies are initiated.

In certain Coprini (2, 8), Uredinales (5), Nidulariaceae (14), and Pyrenomycetes (9, 10, 11, 12), there is evidence that a minus nucleus, when it enters a plus hypha, instead of conjugating with a plus nucleus may travel along that hypha and throughout the plus mycelium. For example, when a plus strain of *Gelasinospora tetrasperma* or *Neurospora tetrasperma* is paired with a minus strain on an agar plate, sometimes the perithecia instead of being limited to the line of contact between the two strains are abundant all over one or both strains (12, 13). Since *G. tetrasperma* produces no secondary spores that might be carried from one strain to the next, and since it has been shown that one strain will not overgrow the other, a scattered distribution of the fruit bodies indicates that nuclei of one sex have travelled out of the mycelia in which they were formed and through the mycelium of opposite sex.

Savile (21) believes that among the Uredinales, nuclei travel from one part of a mycelium to another and that wherever the nucleus is passing through a narrow pore it is fluid and very easily distorted. Mycological literature contains drawings of elongate nuclei in narrow sterigmata of spores. For example, in photographs published by Shaw (22), the wide germinating spores of *Septoria* contain spherical nuclei, but the narrower germ tubes growing from them contain elongate ones.

The study of the fungal nucleus is difficult because there is no agreement among workers about the nature of its organelles. Thus in the yeast according to Lindegren (18) the most conspicuous densely chromatic Feulgen positive

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spherical object in the cell is interpreted by Guilliermond as the nucleus, by Wager and Peniston as the nucleolus, and by Lindegren himself as the centrosome. Furthermore the nuclei of *Gelasinospora* have been observed to flow together to form a mass in which one nucleus is sometimes indistinguishable from another (Fig. 28).

The investigations upon nuclear migration in *Gelasinospora tetrasperma* will be dealt with under the following headings.

1. Migration from One Mycelium into Another Involving Two Sex Factors and One Growth Factor.

In previous work using paired strains of opposite sex to demonstrate nuclear migration (12) mycelia were selected which when mated fruited abundantly. To determine whether nuclear migration would occur in poor-fruiting combinations, two strains of opposite sex were employed which in previous pairings reacted poorly. There was no scattered distribution of fruit bodies to suggest nuclear migration. Therefore experiments were carried out upon mated cultures to determine whether or not nuclei migrate from one mycelium into the other.

2. The Path of Nuclear Migration.

In the experiments mentioned above, tufts of aerial mycelium were removed at the moment that experimental evidence suggested that nuclei might be migrating through them. These were fixed and stained to determine the position of the nuclei in the cells.

3. Migration of the Nucleus Within Its Own Mycelium.

A series of observations were made upon living hyphae to learn whether or not a nucleus can be actually seen in the act of travelling out of one cell and into the next.

Materials and Methods

(1) Selection of Strains for Pairing Experiments

Nine dwarf-sized ascospores of *Gelasinospora tetrasperma* were germinated on malt agar. On slants of the same medium, matings were made between these ascospores to determine their sex. They fell into two groups, four strains being plus and five minus. Then similar pairings were made of these strains, this time on agar plates. About ten days later three matings produced perithecia over one or both strains but the remaining six did not do so even after several weeks.

At first it was assumed that poor fruiting pairs had been cultured upon unsuitable medium or under poor environmental conditions. However this is unlikely. Experiments in this laboratory suggest that rapid and abundant fruiting of paired strains of *G. tetrasperma* is due to heterokaryotic vigor which depends upon the association not only of plus and minus sex factors but also of *F* and *f* alleles. We have poor fruiting when we have the association of plus and minus sex factors but of only one of the *F f* alleles.

Of the poor-fruiting combinations of mycelia of opposite sex, the pair 6 (minus) × 10 (plus) was selected. It was used to test nuclear migration from one mycelium into another one which contained the same allele.

(2) Staining of Nuclei Presumed to be Migrating

Aerial hyphae were floated out on a drop of doubly glass-distilled water on grease-free slides and fixed in 10–15% methyl alcohol. They were either stained lightly with filtered May–Grunwald stain (17) when they were viewed with contrast-phase illumination in order to distinguish the organelles, or with 1 : 15 aqueous Gurr's Improved Giemsa R 66. They were washed in water, dried in air, and permanently mounted in "Piccolyte" (1).

(3) Observations upon Living Hyphae

A minute inoculum from a rapidly growing culture of *Gelasinospora tetrasperma* was placed on a cover slip in a small drop of either sterile water or broth with human serum. The cover slip was placed over water to form a Van Tieghem cell, and incubated at 24° C. for less than 24 hr. For microscopic examination of nuclei, the cover slip, which had been ringed with vaseline, was removed and applied to a glass slide so that the vaselined rim of the cover slip sealed the mount.

1. Migration from One Mycelium into Another Involving Two Sex Factors and One Allele

Experiment 1

Petri plates 15 cm. in diameter half filled with malt agar were inoculated at two points 5 cm. apart with No. 6 (minus) and No. 10 (plus) strains. When the mycelia had grown out and come into contact in the center of the plate, they were allowed to develop further for another four hours. Then, by the aid of millimeter scales drawn outwards from the center on the under side of the dish, small tufts of aerial mycelium were removed from each culture at measured distances from the area of contact between the two strains.

From mycelium 10 (plus), 11 minute tufts of aerial mycelium were taken, six of them from its periphery at distances of 10, 20, 30, 40, 45, and 50 mm. from the center of the plate, five of them at similarly measured distances through the middle of the mycelium.

Each of the 11 tufts was planted separately on slants of malt agar. Within less than a week 10 of them developed perithecia.

Experiment 2

The above experiment was repeated, this time nine tufts of mycelium being transferred, four of them from the periphery of the mycelium at 10, 20, 30, and 40 mm. from the center of the plate, and five of them from a line along its center at measured distances from the center of the plate. This time all transfers fruited.

Conclusion

In experiments 1 and 2, most of the sterile plus mycelium became converted into a fertile condition within four hours after it had reached the minus mycelium. Therefore nuclei migrate into mycelia of opposite sex whether or not they contain a different *Ff* allele. Calculation based on the direct distance from the minus mycelium to the transferred tufts of hyphae shows that minus nuclei must have travelled through the plus strain at the rate of at least 10.5 mm. per hour, more than three times the growth rate of the mycelium from which they came.

These methods might be used to determine whether nuclei will migrate from one mycelium to another of the same sex but with different somatic factors, such as those for color, texture, or pathogenicity.

2. The Path of Nuclear Migration

At measured distances from their point of contact, aerial tufts of hyphae were removed from paired mycelia at the moment that it was calculated nuclei were moving through them. These tufts were fixed and stained.

The majority of the hyphae were filled with cytoplasm and their nuclei were arranged peripherally against the cell wall (Fig. 20). Certain much wider trunk hyphae were empty except for cytoplasmic strands extending straight down the middle of the cell from one septal pore to the next. There were no nuclei near the walls; all were in the central cytoplasmic strands (Fig. 19). The wider hyphae were probably the ones through which nuclei had been travelling.

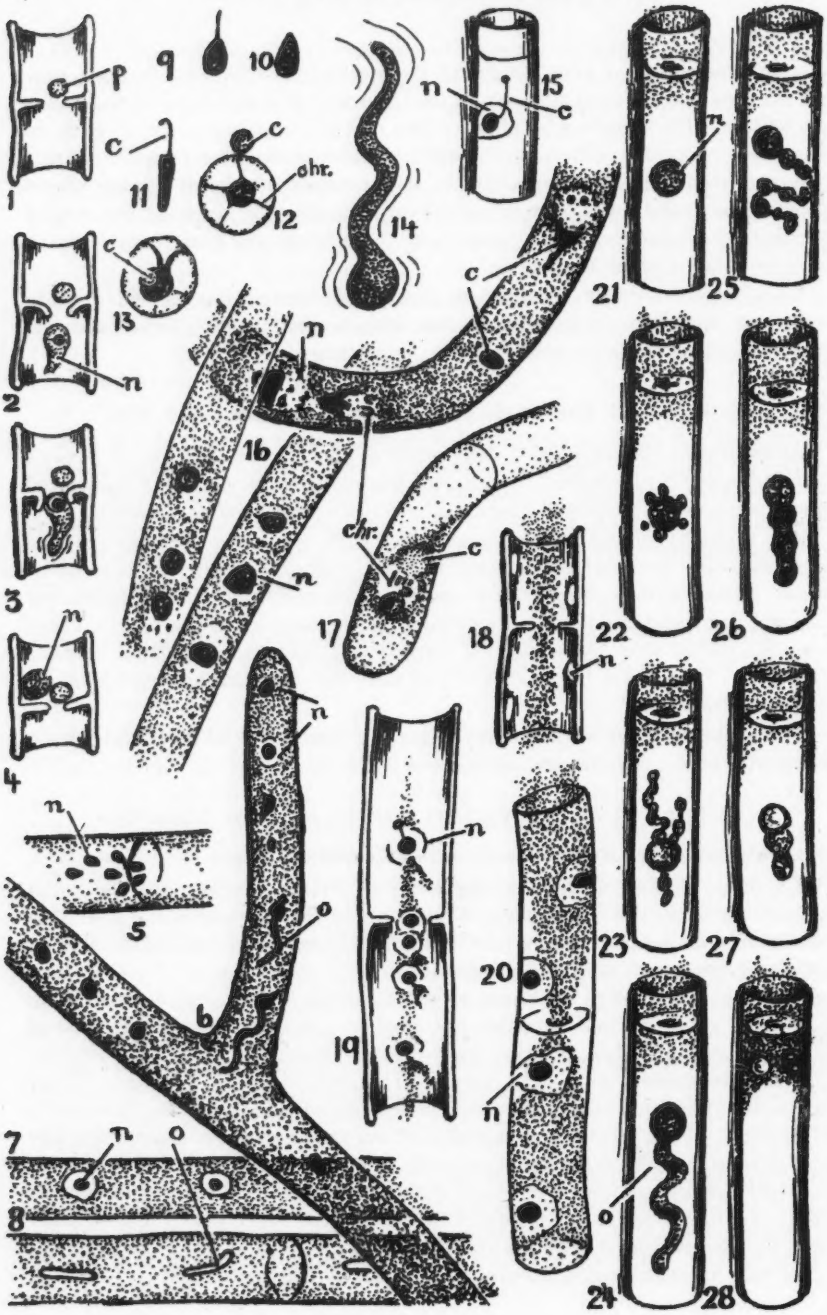
These observations suggest that migrating nuclei are to be found within the center of the cell and not against the walls.

3. Migration of the Nucleus Within Its Own Mycelium

Preliminary Studies of Nuclei in Killed and Fixed Material

The large diploid nucleus in the ascus of *Peziza repanda* and the small haploid nuclei in the mycelium of *Gelasinospora tetrasperma*, in the conidium of *Allescheria boydii*, and in the oidium of *Geotrichum candidum* and of *Trichophyton verrucosum* were all studied.

Pieces of fruit-bodies of *Peziza repanda* bearing young asci were fixed in Flemming's fixative half-strength, bleached in chlorine in sunlight, washed in 95% alcohol and then in water, stained with Haidenhain's iron hematoxylin, and counterstained with light green. This was done in Professor Dame Helen Gwynne-Vaughan's laboratory when one of us visited the University of London. Figs. 29–31 show nuclei in *P. repanda* asci. For a similar study of *Gelasinospora* and other fungi , mycelium was grown in Van Tieghem cells. Some of the preparations were fixed in methyl alcohol and stained in Giemsa (Figs. 6, 16, 32, and 33). Others were fixed by air-drying and immediately permanently mounted without staining (Figs. 9–13, and 15). Fixation by air-drying has been used in the study of mitosis by Darlington and La Cour (7).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28

From an examination of this material it was concluded that fungal nuclei exist in any one of three forms. (*a*) Large nuclei of a diameter almost that of the hyphae. These nuclei are composed of a deeply stained angular or cone-shaped body which is surrounded by or attached to a nuclear vacuole (Figs. 16 and 32). Filaments can sometimes be distinguished in these vacuoles. These nuclei are described as "expanded" (6, 21). (*b*) Small oval nuclei with a diameter of less than a third of the hyphae, densely stained and possessing no nuclear vacuole. Some preparations showed these nuclei arranged about septal pores suggesting they were about to pass through them (Fig. 5). They are described as "contracted." (*c*) Elongate, sinuous, nonvacuolate, deeply-stained bodies bulbous at one end, longer than the width of the hypha. Because of their shape they are called serpentine nuclei or "ophioplasts" (Figs. 6, 14, and 33).

Living Nuclei

In the living hyphae of *Gelasinospora tetrasperma*, there are three types of nuclei: (*a*) expanded; (*b*) contracted; (*c*) serpentine. In the living hyphae of *Allescheria boydii* and of *Penicillium cyclopium* expanded and serpentine nuclei have been observed. The origin of the ophioplast of *Gelasinospora tetrasperma* may be partly nuclear and partly cytoplasmic (4, 16), (Figs. 21–28).

Nuclei During Cytoplasmic Streaming

Observation 1

Under bright field illumination with careful adjustment of light and prolonged watching, cloudlike cytoplasm was seen flowing through the hyphae. One part of a cell containing three nuclei was left under observation for an hour. The streaming continued during the whole time but the three nuclei remained unmoved (Figs. 18 and 20).

Observation 2

A similar preparation viewed with contrast-phase illumination contained a branching hypha with five nuclei. Cytoplasm streamed through the middle of the branch through a septum and into the main hypha, carrying with it amoeboid vacuoles and mitochondria of changing shapes. The five nuclei were arranged against the cell wall, and they did not move from it during the first half hour. Then the cytoplasmic stream reversed. Four of the nuclei moved a distance of less than a cell width and returned again, then they

FIGS. 1–28. Nuclei in *Gelasinospora* mycelium. FIGS. 5, 6, 16, 19, fixed and stained; 9–13, fixed but unstained; all others living. Hypha drawn in Fig. 6 photographed at a different focal plane in Fig. 33. Those drawn in Fig. 16 photographed in Fig. 32. FIGS. 1–4, expanded nucleus approaching and passing through a septal pore; 5, seven contracted nuclei approaching a septal pore; 6, two elongate nuclei (ophioplasts) at base of a branch; 7 and 8, transformation of two expanded nuclei to elongate form during air drying; 9–11, contracted nuclei; 12, 13, 15, and 20, expanded; 14, 24, and 25, elongate; 18 and 20, cytoplasmic streaming leaving nuclei anchored to cell wall; 19, five expanded nuclei fixed while migrating through the center of a hypha; 21–28, an expanded nucleus in a vacuolated cell enlarging to an ophioplast and then contracting and finally becoming indistinguishable from the cytoplasm; *c*, centrosome (in 11 and 15, centriole); *chr*, chromosome; *n*, nucleus; *o*, ophioplast; *p*, plug of septal pore.

continued to move back and forth as though swinging on elastic bridles. Eventually they returned to their original position where they remained for the next 45 min. when the observation was discontinued.

It is concluded that nuclei are not necessarily carried by cytoplasm as it streams through the mycelium, but usually remain anchored to the cell wall.

Migration of Living Nuclei

1. Five nuclei were observed a few cells away from a hyphal tip. Each nucleus was composed of a dark sphere surrounded by, or perhaps attached to, a vacuole. The whole nucleus, sphere and vacuole, possessed a diameter almost the width of the hypha. Sometimes a few radially arranged filaments could be seen in the vacuole.

After two hours one of the nuclei seemed to burst, that is to say, two or three drops of liquid escaped from the nuclear vacuole into the cytoplasm leaving the nucleus oval, contracted, and nonvacuolated. Immediately this nucleus travelled through the cytoplasm to the septum and passed through the septal pore into the next cell. Three similar contracted nuclei followed and passed through the septum and along the hypha.

Next, one of the nuclei with a large vacuole, an expanded nucleus, passed along the hypha and came to rest at a septum. The vacuole gradually poured itself through the perforation with its dark spherical body following behind. This nucleus remained expanded but lost its amoeboid form and became spherical.

2. An expanded nucleus composed of a dark sphere and a tear-shaped vacuole was observed to approach a transverse septum whose pore was closed with a clear spherical plug of material. Ten minutes later the septum became depressed toward the nucleus and then the nucleus passed through the septal pore, sliding past the plug, dark sphere first, the vacuole following with a convulsive movement. It rounded itself off within the adjacent cell, with its sphere now fading from view (Figs. 1, 2, 3, and 4). There was no further change during the next hour.

3. An oval contracted nucleus was observed for half an hour as it moved across the cell and back again to its original position. It suddenly changed its shape into an elongate writhing object about twice as long as the hypha was wide, with one end enlarged slightly, i.e. an ophioplast. The tail part lashed and spun about for seven minutes and then just as suddenly, the ophioplast changed to a short cylinder with a longitudinal thickening. As the cylinder shortened still more, another thickening appeared on its opposite side. It rolled about for four minutes and came to rest, once again an oval contracted nucleus, attached to the cell wall in its original position.

FIGS. 29–33. Nuclei fixed and stained. FIGS. 29–31, *Peziza repanda* expanded diploid nuclei of ascus. Each contains chromosomes and nucleolus and possesses extranuclear chromatin (centrosomes). FIGS. 32 and 33, *Gelasinospora tetrasperma*, haploid nuclei of mycelium. FIG. 32 is drawn and described in Fig. 16. In the looped hypha some of the densely stained centrosomes are attached to expanded vacuolate nuclei with chromosomes. FIG. 33 is drawn at a different focus and described in Fig. 6. It shows a serpentine nucleus. Magnification 1200.

PLATE I

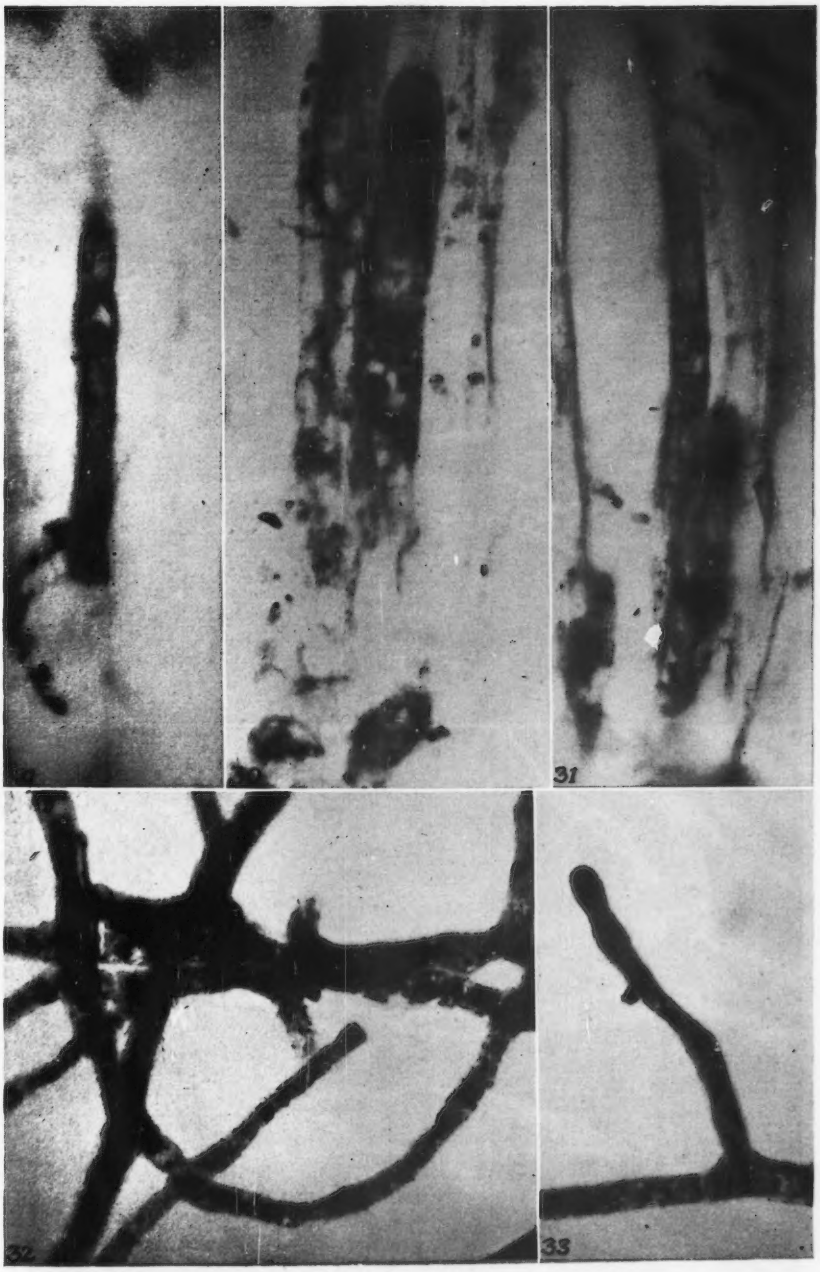
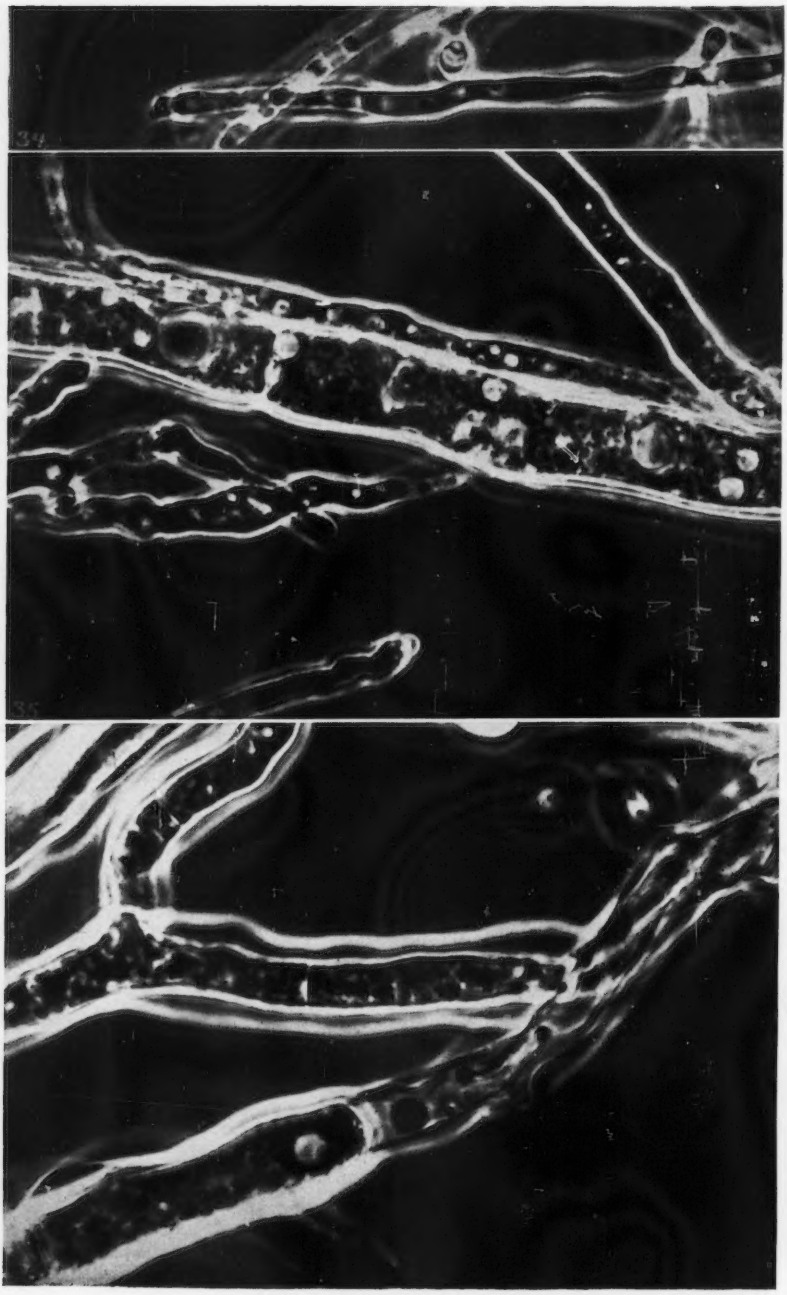


PLATE II



Three more observations were made of an oval unexpanded nucleus becoming converted into an ophioplast then shrinking back to the contracted form.

4. Immediately a preparation of living hyphae was brought into focus under phase-contrast illumination, five or six long ophioplasts, all of them moving sinuously, travelled swiftly through a hypha and disappeared from the field of view.

It may be concluded that the nucleus of *Gelasinospora* exists in any one of three states, expanded, contracted, or serpentine and that it can change from one state to the other (Figs. 7 and 8). Nuclei migrate through the mycelium while expanded, contracted, or serpentine.

Discussion

Migration a Sporadic Phenomenon

The nuclei of fungi may travel from cell to cell within a mycelium or even out of one mycelium and into another. It is estimated that during one month about 50 hr. were spent in observing living nuclei. In all that time nuclei were seen to migrate through the hyphae on only five occasions. Therefore we cannot describe migration as continuous.

Nor are the nuclei evenly distributed throughout the mycelium. In *Gelasinospora tetrasperma* and in *Rhizoctonia solani* the average number of nuclei in a cell of the mycelium is about five. In certain parts of the mycelium the nuclei are more concentrated than this. Examples of such reservoirs are: the germinal vesicle of the ascospore of *G. tetrasperma*, the base of hyphal branches, and the base of the primordial coil in the developing perithecium. Dr. G. B. Sanford and Mr. W. P. Skoropad of the Dominion Laboratory of Plant Pathology counted nuclei in over 600 cells of young *Rhizoctonia solani* mycelium, with the result that they found a concentration of nuclei in T-shaped cells at the bases of the hyphal branches (personal communication). These reservoirs of nuclei are presumably drawn upon at such times as they are needed.

In the development of a fungus, therefore, five nuclear migrations are envisaged: (1) in the young mycelium after the main hyphae have attained their full length; (2) when the lateral branches have developed; (3) after the hyphae have reached and fused with other mycelium of the same species; (4) during the development of the fruit body; (5) after the formation of spores.

FIGS 34–36. Living *Gelasinospora* nuclei. FIG. 34, vacuolated hypha containing two dark bodies interpreted as contracted nuclei. The one in the center has attached to it what may be a centrosome. FIG. 35, in the lining of the central vacuole of the wide hypha are five contracted nuclei with centrioles. FIG. 36, the lower hypha is made up of a vacuolated cell (left) and a nonvacuolated cell (right). Within each cell and near the common septum is an expanded nucleus. There is a third expanded nucleus with a centriole attached, in the upper right-hand corner of the photograph. At a focus lower than that of this photograph were three additional equally large expanded nuclei, making a total of six. The nucleus in the empty cell appears granular, the one in the nonvacuolated cell appears vacuolate. The smaller globules in the empty cell are not interpreted as nuclei. Contrast-phase illumination. Magnification 1200.

Earlier Attempts to Explain Nuclear Migration

Attempts have been made to explain the mechanism whereby nuclei travel out of one mycelium and into another of opposite sex.

At first it was thought that the protoplasm of plus and minus strains of heterothallic fungi may possess a naturally different electric potential. Then nuclei would migrate from one to the other owing to cataphoretic force. Electric currents of the order of 1–10 μa. were allowed to run in either direction through two fused strains *x* and *y* of *Neurospora tetrasperma* (13). This treatment did not reverse the natural direction of nuclear migration, which was always into the *y* strain. We therefore have no evidence that nuclei migrate because they are electrically charged.

A second suggestion is that nuclei are carried by cytoplasm as it streams through the hyphae.

It is known that in a growing mycelium, cytoplasm streams toward the hyphal tips (3); in fact a moving picture has been made of the cytoplasm of *G. tetrasperma* streaming from cell to cell carrying the vacuoles with it. It is conceivable that it might also carry the nuclei. However, earlier experiments with *Gelasinospora tetrasperma* gave no evidence of this but suggested that the cytoplasmic stream leaves the nuclei behind (12). Later observations showed that the nuclei that were observed remained stationary in cells with streaming cytoplasm. We may therefore approach the problem of migration from another point of view.

Postulation of a Motile Fungal Nucleus

Nuclear movement is not the result of any change in electric potential or of any observable external force although it may be influenced by such factors as light (12). Observations on ophioplasts and other moving nuclei suggested to the authors that the fungal nucleus itself may sometimes possess independent motility. It is of interest to consider how far our direct observations support the postulate of an independently motile nucleus with its center of motility residing in its centrosome (Figs. 12 and 16).

In the mammalian sperm the centrosome is concerned with nuclear propulsion (15). Although centrosomes are uncommon among plants they do occur in fungi. In the budding yeast the centrosome is associated with the nucleus as it migrates from the mother to the daughter cell (19, 20, 24). Figs. 29–31 show that the large conspicuous diploid nucleus in the ascus of *Peziza repanda* is an elliptic vacuole, within which are a nucleolus and chromosomes. Attached to it are one or two darkly stained centrosomes. The haploid nucleus in the mycelium of *Gelasinospora* in its expanded state is also a vacuole containing chromosomes and here too a centrosome is attached to it (Figs. 12, 13, 17 and 34). Contracted, expanded, and elongate centrosomes are also shown in Figs. 9–15.

In a living hyphal tip of *Gelasinospora tetrasperma* a nuclear vacuole containing a nucleolus and probably five chromosomes was observed. Attached to one end of it was a cone-shaped mass of cytoplasm which elongated

occasionally to form a tail. This was interpreted as a centrosome (Fig. 17).

In an older vacuolated hypha a large nucleus similar to that shown in Fig. 36 was observed in a stationary position within a vacuole (Fig. 21). Four of five elongate bodies, probably mitochondria (4, 16), alternately approached and retreated from it. When surrounding the nucleus, they took the form of spheres; when in the vacuole, they stretched into beaded filaments. Suddenly the filaments coalesced into a single lashing whip which joined the nucleus. Together the two formed a violently moving ophioplast which when extended reached a length of about a third of that of the cell. It contracted to a short cylinder, rotating more slowly. Finally, one hour after the start of the observation, the body entered and merged with the cytoplasm of the cell (Fig. 28). Figs. 22–24 illustrate the development of what is interpreted as the centrosome during the observation, and how it became attached to the nucleus to form an ophioplast. Singleton (23) states that stained preparations of the ascus of *Neurospora crassa* suggest that an enormous increase in size of the centriole occurs during anaphase.

On the basis of the above observations it is suggested that the nuclei of *Gelasinospora tetrasperma* when they become freed from the cell wall to which they are attached may be capable of independent motility.

Acknowledgments

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THE HEMAGGLUTINATION REACTION TO BCG AND VOLE BACILLUS VACCINATIONS[1]

By Roma Z. Hawirko

Abstract

The antibody response in four groups of five rabbits, three vaccinated with BCG and one with the vole bacillus, was measured by the hemagglutination reaction. In the control series, in which the standard BCG vaccination procedure was carried out, a mean titer of 1 : 16 or higher appeared on the third week and was maintained for eight weeks. In the second series, in which the vaccine was administered in two doses, with a three-week interval, a titer of 1 : 16 or higher appeared on the fourth week and was maintained for 11 weeks. In the third series the vaccine was prepared in a water-in-oil emulsion and administered as in the control series. A titer of 1 : 16 or higher appeared on the second week and was maintained for 13 weeks. In the fourth series the standard procedure was followed, except that the vole bacillus was substituted for BCG. A titer of 1 : 16 appeared on the fifth week and was maintained for three weeks. The differences between the results in series three and in series four and those of the control were significant, whereas the results in series two did not differ significantly from those of the control.

Introduction

The hemagglutination reaction of Middlebrook and Dubos (3) in which a water-soluble fraction from tubercle bacilli is absorbed on erythrocytes so that sensitized cells agglutinate in the presence of antibody to tubercle bacilli, has been extensively studied as a diagnostic tool with inconclusive results (4, 6, 7, 14). Since the antibody responsible for the hemagglutination reaction is circulated in the blood of immunized animals, the reaction presents a new approach for assessing the response of the animal body to BCG vaccinations. However, there have been few publications on this subject (4). While the hemagglutination test may not give a measure of the protective effects of antibody, it does seem to provide a quantitative test for the determination of the antigenic potency of mycobacterial vaccines. The present report deals with the course of hemagglutinin levels in rabbits vaccinated with BCG and with the vole bacillus.

Materials and Methods

In order to obtain a high percentage of viable microorganisms and eliminate the effects of dead bacilli, the BCG vaccines were prepared from a 10-day growth of strain 867S-2 in tween-albumin medium of Dubos and Middlebrook (3). The cells were washed and resuspended in Sauton's medium diluted to one-quarter strength to contain 30 mgm. dry weight bacillary bodies per ml.

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All BCG and vole bacillus vaccines were used two days after preparation. Culture assays prepared by plating serial dilutions of the vaccine showed approximately 10^9 viable bacterial units per ml. concentrated suspension.

The BCG administered in two doses with a three week interval consisted of the 30 mgm. per ml. suspension as in the control series. Each of the two multiple puncture doses which are usually given simultaneously were administered separately and accordingly the total amount of vaccine remained the same. The vaccine for the second dose was prepared from a fresh 10-day culture.

The water-in-oil emulsified vaccine was prepared from a 0.75 ml. suspension of BCG in 3.0 ml. light mineral oil and 0.75 ml. Falba, a lanolin derivative which served to stabilize the emulsion. The dry weight of the tubercle bacilli in the emulsified vaccine was 3.0 mgm. per ml., which was 1/10th the amount used in the aqueous BCG vaccines.

The vole bacillus vaccine was prepared from a heavy growth of *Mycobacterium muris*, strain OV 20572, which was cultured in a tween–albumin medium with succinic acid as the sole source of carbon described by Ackart and Murray (1). At four weeks the culture produced a growth density comparable to that at 10 days with BCG. The cells were washed and resuspended in sufficient phosphate buffered saline, pH 7.0, to yield 5 mgm. dry weight bacillary bodies per ml.

Five rabbits were vaccinated in each of the four series by the multiple puncture disc method of Rosenthal (10) with scarification on both sides of the vertebrae. In the first or control series the standard BCG vaccination procedure was followed. In the second series the vaccine was administered in two doses: the initial vaccination on one side of the spinal column was followed in three weeks by a second dose on the other side. In the third series the vaccine was prepared in the water-in-oil emulsion and administered as in the first series. In the fourth series the standard procedure was followed except that the vole bacillus was substituted for the BCG.

Tuberculin tests, carried out prior to vaccination with 10 mgm. Old Tuberculin, were negative. Serum samples were obtained prior to tuberculin testing and vaccination and at weekly intervals following vaccination for 11 to 17 weeks. The sera were stored at about $-20°$ C. and thawed at room temperature on the day of testing. The hemagglutination test was carried out as described (8) with the following modifications. The antigen used for the sensitization of the sheep cells was a dilution of human Old Tuberculin, 25%, received from the Animal Diseases Research Institute, Hull, Quebec. It was diluted 1 : 60 in phosphate buffered saline, pH 7.0, to give concentration equivalent to Lederle's 4× tuberculin diluted 1 : 12. Serum dilutions in 0.4 ml. buffered saline were used with an equal volume of 0.2% sheep cells. Hyperimmune serum for positive control, prepared by injection of heat-killed bovine tubercle bacilli (Ravenel) as described by Rothbard and associates (11) gave a titer of 1 : 1024.

Results

The mean hemagglutination titers, or highest dilutions of sera showing agglutination of the sensitized cells, in the four vaccination series are presented in Table I. A titer of 1 : 16 or higher appeared on the third week following vaccination with BCG in the control series, on the fourth week in the series in which the vaccine was administered in two doses, and on the second week when the vaccine was prepared in the water-in-oil emulsion. Vaccination with the vole bacillus did not attain a titer of 1 : 16 until the fifth week. The duration of a titer of 1 : 16 or higher was maintained for eight weeks in the control series; 11 weeks in the second; 13 weeks in the third; and three weeks in the fourth (Table II).

TABLE I

MEAN HEMAGGLUTINATION TITERS IN RABBITS FOLLOWING VACCINATION WITH MYCOBACTERIA

Weeks	Vaccination series			
	I BCG*	II BCG	III BCG	IV Vole
0	0	0	4**	4
1	0	0	8	4
2	4	8	16	8
3	32	8	32	8
4	32	16	32	8
5	64	32	32	16
6	32	32	64	16
7	64	32	64	16
8	32	64	64	8
9	16	32	32	4
10	16	32	32	4
11	8	32	32	4
12	4	16	32	—
13	4	16	32	—
14	—	16	16	—
15	—	8	4	—
16	—	8	4	—
17	—	—	0	—

** I standard procedure; II two vaccination doses; III with adjuvant.*
*** Titer recorded in terms of reciprocal serum dilutions, 0 = reaction, — test omitted.*

TABLE II

DURATION OF HEMAGGLUTINATION TITERS FOLLOWING VACCINATION

Titers	Vaccination series			
	I	II	III	IV
0-1 : 4	3*	2	1	2
1 : 8 or higher	9	15	14	7
1 : 16 or higher	8	11	13	3
1 : 32 or higher	6	7	11	0

* *Time in weeks.*

Although individual rabbits in the BCG vaccinated groups showed titers as high as 1 : 128 to 1 : 256, the highest mean level obtained was 1 : 64, which was observed between the fifth and eighth weeks. In the vole vaccinated rabbits the highest individual titer recorded was 1 : 32 and the highest mean level of 1 : 16 also occurred between the fifth and eighth weeks.

Discussion

In the present study the titer of the sera of all vaccinated rabbits increased to 1 : 16 or higher as measured by the hemagglutination reaction. Recently, Fleming *et al.* (4) and others suggested that the titer must be definitely greater than the previously accepted level of 1 : 8 (8, 13) to serve as the line between the significant and nonsignificant reactions. For this reason a titer of 1 : 16 or higher was selected as indicating a significant response. However, essential differences between the control series and the other series remain the same regardless of whether a titer of 1 : 8, 1 : 16, or 1 : 32 is selected as the line of demarcation (Table II).

The observations in the control series are in agreement with those of other workers (4, 8). In the series in which the BCG vaccine was administered in two doses the response was greater than in the control series, but the difference was not significant statistically as shown by t test. It would seem that the apparent increased response was due to the secondary antigenic stimulus. Raffel (9) reports that the advantages of the secondary stimulus are not always evident since the stimulus depends not alone upon the level of antibody but largely upon the training of the antibody forming cells and their consequent ability to respond quickly and effectively to a challenging infection. It would appear that protection tests are required in order to assess the effect of a secondary stimulus in BCG vaccination. Although no untoward reactions were observed in the animals, there are serious objections to this method. There is the possibility that the hypersensitive tissue would react to the second vaccination dose.

In the series of rabbits which were vaccinated with BCG suspended in the water-in-oil emulsion the increase in antibody titer was greater and was maintained for a longer time than that in the control series. The differences were shown to be statistically significant according to the *t* test. This result is in agreement with the adjuvant activity of water-in-oil emulsions of antigens with respect to levels and persistence of antibodies in other immune systems (5, 12). It may be noted that the increased response was obtained even though the vaccine dose was reduced to 1/10th of the control dose. The use of a water-in-oil emulsion of BCG would seem to offer a more effective vaccine with the additional advantage of reducing the number of viable microorganisms required to produce a satisfactory antibody response.

The antibody levels following vaccination with the vole bacillus were significantly lower than the control and the response was not maintained for as long a period. The results suggest that immunization with the vole bacillus may be of a low order. This might be attributed to the possibility that the Old Tuberculin used as a sensitizing antigen was more specific for BCG. This view is supported by the finding of Birkhaug (2) that tuberculin sensitivity took longer to develop in guinea pigs vaccinated with the vole bacillus than in a control group vaccinated with BCG. Or it might result from the use of a smaller amount of vole bacilli in the vaccine (5 mgm. per ml. instead of 30 mgm. in the control series). The reduced dosage was used to conform with the amount used by other workers (2, 15). Both Birkhaug and Griffith (2) indicate that the vole bacillus is considerably more virulent than BCG, larger doses causing progressive lesions in experimental animals. The smaller dose of BCG in series three (3 mgm. per ml. instead of 30 mgm.), however, did not appear to affect the antibody level.

It would seem that new vaccination techniques with mycobacterial antigens and the standardization of technical factors such as the size of dose could be studied with advantage by the hemagglutination reaction and should lead to more effective and reliable immunization methods.

Acknowledgments

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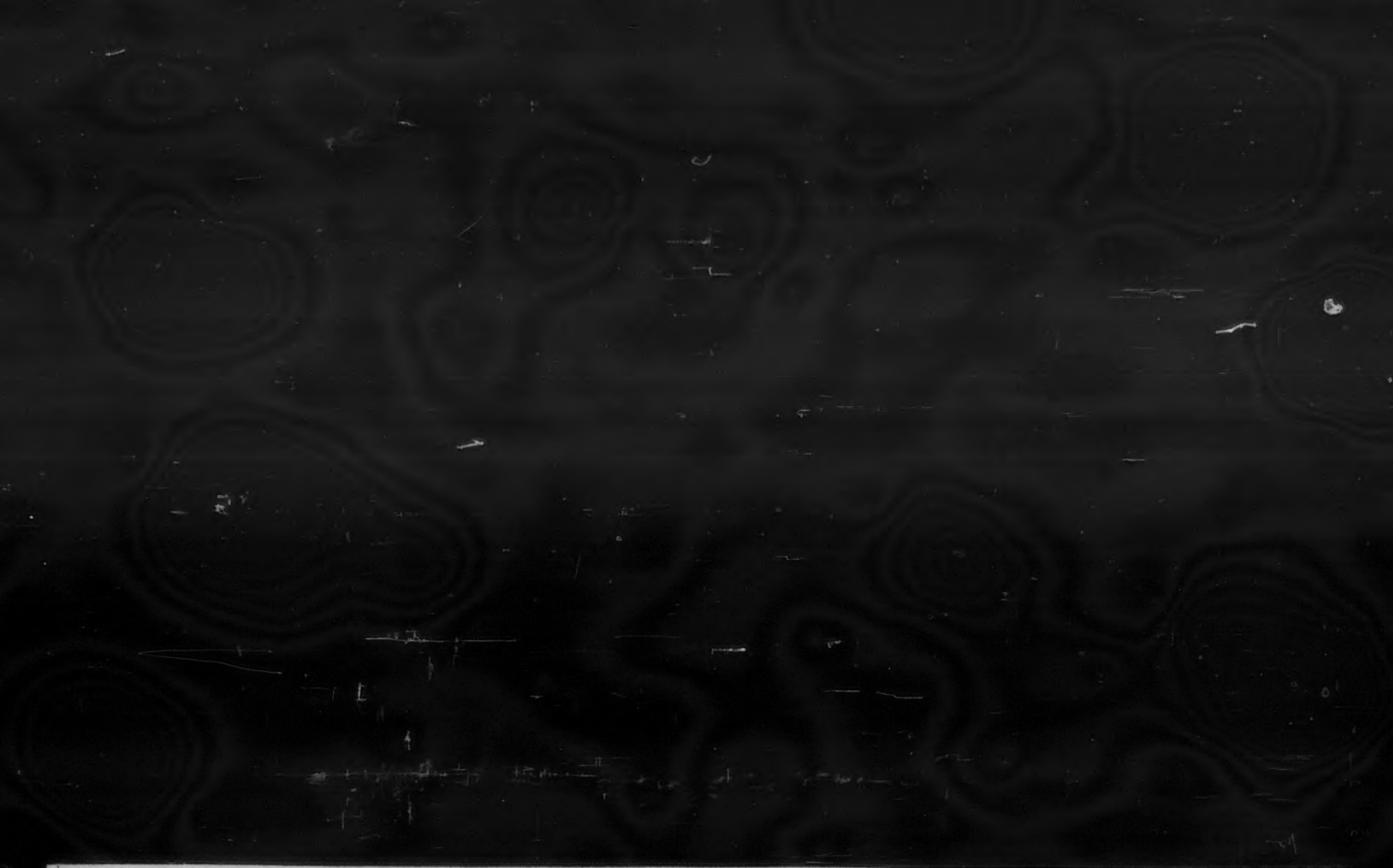
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